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THE INFLUENCE OF SOME HORMONAL, DIETARY, AND TUMOR FACTORS ON LIVER CATALASE ACTIVITY IN RATS[1]

BY R. W. BEGG[2], T. E. DICKINSON[3], AND A. V. WHITE

Abstract

Liver catalase activity can be reduced by the administration of cortisone or stilboestrol and by the production of anemia, as well as by the presence of a tumor in the host. All of these factors cause a disturbance of body growth. However, interference with growth produced by adrenalectomy or low protein diet is not associated with loss of liver catalase activity. The loss of liver catalase activity in tumor-bearing rats is associated frequently with an increase in liver size. But rats with large tumors may have small livers and still demonstrate the drop in catalase activity. It is suggested that the loss of liver catalase activity in tumor-bearing rats is not due to body growth disturbance or liver hypertrophy, and is produced in excess of such nonspecific factors as adrenal stimulation.

Introduction

Consideration of the loss of liver catalase activity in tumor-bearing rats (12) leads to the realization that little is known of the factors that control the level of this enzyme in the livers of normal rats. The secretions of the pituitary (10), the adrenal, and the gonads (1) have been suggested as influencing the level, and studies with labelled iron in the guinea pig have yielded information concerning the synthesis of catalase at different sites in the body (20). Several papers on the relation of dietary protein to catalase activity have appeared (2, 17, 21), but the interpretation of these findings in relation to the tumor-bearing rat is not clear.

In conjunction with studies on the liver catalase level in tumor-bearing rats experiments with normal rats under various experimental conditions have been conducted. The results, and difficulties of interpretation, will be presented here.

Methods

Sprague-Dawley rats maintained on fox chow and tap water were used, except under specified dietary conditions. The high and low protein diets

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are those described by Miller (17). Cortisone acetate[1] was administered by subcutaneous injection. Stilboestrol[2] was given in drinking water at a concentration of 5 μgm. per ml. Rats were adrenalectomized by the lumbar route, and maintained on a high sodium – low potassium diet and saline (8). Anemia was produced by repeated withdrawal of 2 ml. of blood by heart puncture.

Hemoglobin was determined by the method of Evelyn (9), and catalase, in the early experiments, by the method of Greenstein (11) at a concentration of 0.04 mgm. tyrosine per ml. (21) (Method 1). In the later experiments more consistent results were obtained by using the method of Dounce (7) at a concentration of 0.02 mgm. tyrosine per ml. (Method 2).

Tumor weights are expressed as per cent of the total body weight. The term carcass weight refers to the total body weight minus tumor weight. Liver weight is expressed as gm. per 100 gm. total body weight. Results are expressed as means ± the standard error of the mean.

Results

In these experiments adrenalectomy produced a small drop in liver catalase activity, but not to a significant degree. In view of a previous report (6) it may be said that the results in Table I are a compilation of three experiments, in one of which there was a significant decrease in catalase activity. The expected drop in hemoglobin level did occur, with a tendency to restoration by replacement therapy. The adrenalectomized animals did not gain weight as well as the controls, and in an observation period of 12 days the adrenalectomized rats ate a total of 171 ± 4.2 gm. per rat while the controls consumed 205 ± 9.0 gm. Thus, there was no significant loss of catalase activity, despite the presence of an inanition factor. The depression of catalase by replacement therapy was an unexpected finding. Liver weights are not available.

TABLE I

EFFECT OF ADRENALECTOMY ON LIVER CATALASE ACTIVITY

Group	Initial body weight, gm.	Time, days	Δ body weight, gm.	Hemoglobin, gm./100 ml.	Catalase,** K × 10⁴
Control (18)	144	15	+63	14.9 ± 0.3	3899 ± 96
Adrenalectomized (18)	144	15	+35	13.1 ± 0.3*	3618 ± 143
Adrenalectomized plus cortisone‡ (6)	185	7	− 1	14.2 ± 0.7	2622 ± 376†

* *P = <0.01.* † *P = 0.02.* ‡ *0.5 mgm. daily, begun eight days postoperative.*

** *Method 1.*

[1] *Supplied by the National Research Council of Canada.*

[2] *Supplied by Ayerst, McKenna, and Harrison.*

In view of the results obtained with cortisone in the adrenalectomized rat it was administered to intact rats at two dose levels.

Cortisone at a level of 1 mgm. daily produced a significant decrease in catalase activity, with a further decrease at a level of 3 mgm. daily. The expected increase in hemoglobin level was observed, though it should be noted that studies on blood volume were not done. No great variation in liver weight was noted. At the dose levels used a stepwise decrease in growth rate was observed.

TABLE II

EFFECT OF CORTISONE ON LIVER CATALASE ACTIVITY

Group	Initial body weight, gm.	Time, days	Δ body weight, gm.	Liver, gm./ 100 gm.	Hemoglobin, gm./100 ml.	Catalase†, K × 10^4
Control (6)	198	13	+74	4.32	14.2 ± 0.2	6278 ± 80
Cortisone, 1 mgm. (6)	198	13	+41	4.12	15.6 ± 0.3*	5073 ± 219*
Cortisone, 3 mgm. (6)	198	13	+13	4.81	16.5 ± 0.1*	4370 ± 258*

* $P = <0.01$. † *Method 1.*

Experiments with tumor-bearing male rats (to be reported) required that the animals be given the estrogen stilboestrol. To determine the effect of stilboestrol per se on catalase activity it was added to the drinking water of male rats.

TABLE III

EFFECT OF DIETHYLSTILBOESTROL ON LIVER CATALASE ACTIVITY IN THE MALE RAT

Group	Initial body weight, gm.	Time, days	Δ body weight, gm.	Hemo-globin, gm./100 ml.	Catalase†, K × 10^4
Control (5)	209	10	+36	14.1	4585 ± 210
Treated (5)	207	10	−13	14.0	3644 ± 94*

* $P = <0.01$. † *Method 1.*

Stilboestrol caused a moderate drop in catalase activity with no effect on hemoglobin. The estrogen, at this dose level, not only prevents weight increase in male rats, but causes an actual loss of body weight. Liver weights were not recorded. Taking a mean figure of 20 ml. as the daily water ingestion the rats received 100 μgm. of stilboestrol daily.

The tumor-bearing rat exhibiting loss of liver catalase activity is anemic, and both hemoglobin and catalase are heme-proteins. It is possible that anemia might divert most of the building blocks for heme-proteins to hemoglobin, and produce a deficiency in catalase. Anemia was produced by 21 bleedings in 28 days.

TABLE IV

EFFECT OF ANEMIA ON LIVER CATALASE ACTIVITY

Group	Initial body weight, gm.	Time, days	Δ body weight, gm.	Liver, gm./100 gm.	Hemoglobin, gm./100 ml.	Catalase†, K × 10^4
Control (5)	175	28	+107	3.90	15.4 ± 0.1	3066 ± 59
Anemia (5)	178	28	+ 59	4.10	8.5 ± 0.5*	2464 ± 100*

* $P = <0.01$. † *Method 2.*

The production of anemia by the method described produces a moderate loss of catalase activity in the experimental animals. This is not accompanied by an alteration in liver size but by a marked effect on growth rate. The control animals were "sham punctured", but no blood was removed.

To study the relation of liver size to catalase activity the Walker 256 carcinoma was allowed to grow to a large size, representing 27% of the body weight.

TABLE V

MALE RAT BEARING WALKER 256 CARCINOMA

Group	Initial body weight, gm.	Time, days	Δ carcass weight, gm.	Liver, gm./100 gm.	Hemoglobin, gm./100 ml.	Catalase†, K × 10^4
Control (6)	173	15	+55	4.10	14.5	3772 ± 202
Tumor bearer (6)	168	15	+ 5	4.30	8.8	1282 ± 159*

* $P = <0.01$. † *Method 2.*

When observations from the tumor-bearers were pooled, the anemia and loss of liver catalase activity were noted, as well as the failure of increase in carcass weight. The increase in liver size, frequently observed in tumor-bearers (Table VIII), was not present. Individual tabulation of the results in Table VI demonstrates that the animals may be divided into two groups: one shows an actual loss of carcass weight and small livers, the other group gained some carcass weight and had livers larger than normal. In both instances catalase activity was decreased, slightly more in the first group.

TABLE VI

MALE RAT BEARING WALKER 256 CARCINOMA

No.	Initial body weight, gm.	Final carcass weight, gm.	Δ carcass weight, gm.	Tumor, %	Liver, gm./100 gm.	Hemoglobin, gm./100 ml.	Catalase*, K × 10^4
1	164	157	− 7	28	2.51	8.7	690
2	186	182	− 4	27	3.33	7.9	1200
3	154	154	0	29	3.62	9.0	1020
4	180	211	+31	27	5.10	7.6	1430
5	148	152	+ 4	28	5.21	9.0	1630
6	174	179	+ 5	22	5.78	10.6	1720
Mean	168	173	+ 5	27	4.30	8.8	1282

* *Method 2.*

In many of the experiments on tumor-bearing rats loss of body weight is relative rather than absolute, i.e. the tumor rats do not gain as rapidly as controls of the same initial weight, but they do gain. It seemed of interest to graft some plateaued female rats with the Walker tumor and study the effect on body weight.

TABLE VII

PLATEAUED FEMALE RATS BEARING WALKER 256 CARCINOMA

Group	Initial body weight, gm.	Time, days	Δ carcass weight, gm.	Liver, gm./100 gm.	Hemoglobin, gm./100 ml.	Catalase‡, K × 10^4
Control (6)	230	14	0	3.24	15.3	2675 ± 90
Tumor bearer† (5)	230	14	−18	4.46	10.2	1496 ± 96*

* $P = <0.01$. † *22%.* ‡ *Method 2.*

TABLE VIII

EFFECT OF DIETARY PROTEIN ON LIVER CATALASE ACTIVITY

	Initial body weight, gm.	Time, days	Δ carcass weight, gm.	Liver, gm./100 gm.	Hemoglobin, gm./100 ml.	Catalase†, K × 10^4
25% Protein (6)	114	14	+59	5.80	13.3	2522 ± 79
25% Protein, tumor bearer‡ (6)	113	14	+ 8	9.20	7.8	1342 ± 126*
6% Protein (6)	115	14	−12	5.00	15.1	2487 ± 101
6% Protein, tumor bearer** (6)	111	14	−24	6.60	8.7	1170 ± 63*

* $P = <0.01$. † *Method 2.* ‡ *26%.* ** *25%.*

Loss of 8% of the body weight is associated with diminished catalase activity, but to a less extent than is the loss in activity associated with failure to gain weight.

Clear-cut evidence of a lack of correlation between failure to gain in weight and loss of catalase activity is provided by experiments with low protein diets.

The tumor-bearer on an adequate protein diet exhibits diminished gain in carcass weight and marked loss in catalase activity. The nontumor rat on a low protein diet (6%) shows an actual loss of carcass weight, but normal catalase activity. Liver hypertrophy is present to a lesser degree in the protein depleted group.

Discussion

It has been suggested (7, 16) that inanition is a causative factor in the reduced liver catalase activity found in tumor-bearing rats. Force-feeding tumor-bearing rats provides experimental evidence which is not in agreement with this concept. Carcass weight loss did not occur, but the typical catalase depression was unchanged (5). The present experiments tend to confirm the view that there is no causative relation between weight loss and decrease in catalase activity. The correlation between weight and catalase loss is present in many experiments, but in the adrenalectomy group there is a failure to gain weight and no loss of catalase activity. Nontumor rats on a low protein diet exhibit an actual loss of carcass weight, with no concomitant effect on catalase activity.

Miller claims that fasting or a low protein diet produces a loss in catalase activity (17). This conclusion is based on certain theoretical calculations, but when expressed as per unit of protein nitrogen Miller's results are in agreement with those of the present experiments. It appears that catalase is lost from the livers of protein-depleted rats at the same rate as other proteins, and that its concentration remains unaltered.

It has been suggested that the loss of liver catalase activity is a mere dilution effect, the result of liver enlargement (13). This has not seemed an impressive argument, as the liver enlargement is a real increase of protoplasm (19) and it would then be necessary to envisage synthesis of new protoplasm devoid of catalase, i.e. a relative deficiency. Indeed the figures from Klatt and Taylor's publication show a decrease in over-all catalase activity (13). It has been demonstrated that a few days antemortem there is a rapid loss of liver substance (19) but no reference has been encountered suggesting a terminal rise in catalase activity. The data in Table VI indicate that low catalase activity is present in the liver irrespective of the liver size.

The present data are not in agreement with a previous publication which suggested that adrenalectomy decreased catalase activity at the $P = 0.05$ level of significance (6). It would appear that if the absence of the adrenal in the rat has any effect on liver catalase activity it is of a small order of magnitude. On the other hand the administration of cortisone has a definite

depressing effect on activity. If this effect is to be considered in the light of the protein catabolic effect of cortisone, then catalase apoenzyme must be catabolized more readily than other liver protein, in contrast to the effect of low protein diets.

The effects of adrenalectomy and cortisone on the rat described here are in direct contradiction to the effects noted by Adams in mice (1). The only explanation available is that of a species difference. There is precedent for opposite effects in enzyme concentration in different species after the administration of the same hormone. Testosterone causes quite different effects on kidney alkaline phosphatase in the rat and mouse (14).

At the moment it is not clear whether stilboestrol produces a loss of catalase activity by direct action or via the adrenal (15, 18).

If the thesis that catalase depression is not dependent on restriction of somatic growth is true then the effect of anemia on catalase activity is of great interest. It is obvious that tracer experiments in normal and tumor-bearing animals under experimental conditions of caloric restriction, anemia, and other factors described in this paper would yield useful information concerning the turnover rates of catalase and hemoglobin.

Consideration might be given to adrenal stimulation as the factor common to the various experimental states producing catalase depression. A previous suggestion that the tumor-bearing rat is in a state of adrenal hypofunction (3) does not seem tenable in view of the fact that the adrenalectomized tumor-bearing rat does not show thymus involution (4). Concomitant studies on liver catalase activity in adrenalectomized tumor-bearing rats could not be done owing to poor survival rates.

Indirect evidence suggests that while adrenal stimulation may be a factor in many procedures producing a fall in catalase activity, it is not the only factor. If the degree of thymus involution is taken as an index of activity of the adrenal cortex, then involution of 15% of the thymus in the anemic rat is associated with as great a catalase loss as the 45% involution noted in rats receiving 1 mgm. cortisone, and the tumor-bearing rat exhibiting thymus involution to the degree found in the rats given 3 mgm. cortisone (85%) has twice the loss of catalase as the cortisone treated rat.

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THE EFFECT OF BENIGN AND MALIGNANT TUMORS ON LIVER CATALASE ACTIVITY[1]

By R. W. Begg[2], T. E. Dickinson[3], and J. Millar[4]

Abstract

Liver catalase activity has been determined in rats carrying a transplantable benign mammary fibroadenoma, fibroadenomas undergoing malignant change, and a transplantable malignant fibrosarcoma derived from the fibroadenoma. The benign tumor causes a small depression of liver catalase activity. When the tumor undergoes a malignant change there is a sharp decrease in liver catalase activity, not explained by change in liver size or growth rate of the tumor.

Introduction

The diminished liver catalase activity of tumor-bearing rodents is a well documented experimental finding (1, 2, 6, 7). Experiments to date have been conducted on animals bearing malignant tumors compared to nontumor controls. The opportunity arose in this laboratory to study the reaction of the host to the presence of a benign tumor, and to this tumor undergoing a malignant transformation. It seemed that a study of liver catalase and hemoglobin under these conditions might yield information useful in the interpretation of enzyme changes in the livers of tumor-bearing animals.

Experimental

The benign tumor used in these experiments arose spontaneously in a stock female of the Sprague-Dawley strain and has been carried by transplantation. Pathologically it is described as a mammary fibroadenoma (8), with a predominance of fibrous tissue.

On occasion the tumor undergoes spontaneous transformation to a fibrosarcoma. If allowed to grow it produces cachexia in the host, and subsequent death.

In tumors subject to malignant transformation the malignant area may be differentiated from the benign tissue in the gross, and transplants of the malignant areas have been made. These give rise to a typical transplantable fibrosarcoma. Thus, there is available for experimental purposes (*a*) the benign fibroadenoma, (*b*) a mixed fibroadenoma–fibrosarcoma, and (*c*) the transplantable fibrosarcoma, all of which have arisen from the original spontaneous fibroadenoma or its subsequent transplanted generations.

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The benign tumors used in this study were dependent on estrogens, i.e. they would only grow in the male treated with estrogen. For this purpose males were maintained with stilboestrol given in the drinking water at a concentration of 5 μgm. per ml.

The tumors were transplanted into Sprague-Dawley females or males maintained on estrogen. Fox chow was used as a diet throughout.

Prior to sacrifice, hemoglobin was determined on tail blood by colorimetric method (3). The rats were killed by cervical dislocation, the livers removed, washed, chilled, and homogenized in the Waring Blendor with three volumes of distilled water. Tumors were dissected free and weighed. All differentiation of benign and malignant tumors was checked by histological examination.

Catalase was determined on the liver homogenate by the method of Greenstein (5), the homogenates being diluted to a concentration of 0.1 mgm. *N* per ml. Results are expressed as the mean ± the standard error of the mean.

Results

The two most striking figures in Table I are the demonstration that a rat bearing a benign tumor equal in weight to the host produces only an 8% drop in catalase activity, while a tumor of equal size which is a mixture of benign and malignant cells produces a fall of 44% of the catalase activity. A smaller, but entirely malignant tumor, produces a slightly greater fall in catalase activity.

TABLE I

TUMOR-BEARING FEMALE RATS

Group	No. of rats	Tumor, % body weight	Hemoglobin, gm./100 ml.	Catalase, K × 10^4
Control	6	—	14.7 ± 0.2	2842 ± 33
Benign tumor	7	51	11.4 ± 0.8*	2609 ± 69†
Benign–malignant tumor	4	49	8.9 ± 1.0	1600 ± 140‡
Malignant tumor	7	30	9.1 ± 1.1	1353 ± 175

* $P = < 0.01$ *when compared with control.*
† $P = < 0.02$ *when compared with control.*
‡ $P = < 0.01$ *when compared with benign.*

The anemia in the host of the benign tumor is more striking than the loss of catalase activity, 22% of the hemoglobin being lost in contrast to 8% of the catalase. The mixed and the malignant tumors produced an anemia of a lower mean value, but not significant owing to large variation.

In the male rat the depression of catalase activity is more striking than in the female, 38% of the activity having been lost, while the loss of hemoglobin was 30%.

TABLE II

TUMOR-BEARING MALE RATS

Group	No. of rats	Tumor, % body weight	Hemoglobin, gm./100 ml.	Catalase $K \times 10^4$
Control	10	—	15.3 ± 0.3	4119 ± 109
Benign tumor†	9	38	10.6 ± 1.0*	2549 ± 283*

* $P = < 0.01$.
† *Treated with diethylstilboestrol (see Ref. 1).*

The stilboestrol required to produce tumor growth in the male rat causes a loss of 20% of liver catalase activity (1).

Thus it appears that the depression of catalase activity in the male rat bearing the benign tumor is a summation of the effect of the tumor and the effect of the estrogen. It is of interest that while the estrogen did not produce any anemia the estrogen treated tumor-bearing male rat had lost 30% of the hemoglobin. This again directs attention to a more marked effect on hemoglobin than on catalase by the benign tumor.

The liver weights of the rats have been expressed as grams per 100 gm. body weight, i.e. host plus tumor, and per 100 gm. carcass weight, i.e. body weight minus tumor weight.

TABLE III

LIVER WEIGHT OF FEMALE RATS

Group	No. of rats	Mean liver weight	
		Per 100 gm. BW*	Per 100 gm. CW**
Control	6	3.6	3.6
Benign tumor	7	3.1	6.3
Benign–malignant tumor	4	3.1	6.2
Malignant tumor	4	3.7	5.4

* *BW — body weight.*
** *CW — carcass weight (body weight minus tumor).*

It will be seen from Table III that as the tumor increases in size so does the liver, and expressed on a body weight basis there is no liver hypertrophy. However, if the weights are expressed on a carcass weight basis then all tumor bearers have larger livers than the controls. Attention is directed to the fact that whereas there is a marked difference in the catalase activities of the benign vs. the benign–malignant groups (Table I) the livers of these two groups are of the same size.

An attempt has been made to provide data relevant to the question of the effect of growth rate of a tumor on catalase depression. The benign fibroadenoma may be retarded in growth by the administration of moderately large doses of diethylstilboestrol, in this instance 25 μgm. daily in oil.

TABLE IV

GROWTH OF TUMORS

No.	Growth period, days	Tumor weight, gm.	Tumor, % body weight	Catalase, K × 10^4
Benign tumors				
1	180	214	46	2450
2	150	260	50	2450
3	240	345	58	2960
4	60	210	47	2650
5	45	192	46	2600
6	60	389	64	2475
Benign–malignant tumors				
1	260	281	53	1520
2	210	202	47	1240
3	210	241	52	1800
4	200	225	44	1840

In the benign tumors there is no difference in catalase activity despite sharp differences in growth rate. The benign–malignant group demonstrates a marked difference in catalase activity when compared to the benign group of comparable growth rate.

Discussion

It may be objected that conclusions are being drawn from experiments involving small groups, particularly the benign–malignant group. The differences are clear-cut, and the statistical analyses give some correction for the size of the groups. Originally data from 6 control female rats, 17 benign, 6 benign–malignant, and 9 malignant tumor-bearers were available. Some of these rats were rejected from the final analyses as they had been subjected to hormonal treatment for other experimental purposes. It may be said that, though they were rejected, inclusion in the final data would not have changed the conclusions.

The difference in catalase depression would be less impressive when comparing the benign and malignant tumors if there were sharp differences in growth rate (6). The malignant tumors require 30–45 days to achieve 30% of the body weight, whereas many benign tumors require 180 days. Under these circumstances the benign tumors would be making less demand on the

host per unit of time, and the host could maintain homeostasis. But it has been demonstrated (Table IV) that benign–malignant tumors with the same growth rates as some benign tumors have greater effect on liver catalase activity, and that some benign tumors with growth rates comparable to the malignant tumors do not have the same degree of catalase depression.

Catalase depression in tumor-bearing rodents has been dismissed as nonspecific, as a similar reaction can be produced by such divergent procedures as total body irradiation in mice (4) and the presence of leproma in rats (2).

It has been demonstrated that while diethylstilboestrol can cause catalase depression (1), the addition of a benign tumor to the equation causes a further depression. The "specific" factor in a tumor may prove to be an inhibitor which combines with the iron-porphyrin group (7), and is present in higher concentration in malignant tumor, while the nonspecific reactions may be concerned with a relative loss of apoenzyme (1).

The question of the relation of liver hypertrophy to catalase depression has been discussed (1). In the results presented here it is obvious that liver enlargement is not a factor. The liver weight per 100 gm. body weight is the same when comparing control and malignant groups. The benign and benign–malignant groups have identical liver sizes irrespective of the method of expressing them, but the catalase activities are of a different order.

The reason for the apparent difference in the effects on hemoglobin and catalase is not clear at the moment. Though both compounds are hemeproteins the rate of turnover of radioiron by hemoglobin and liver catalase is quite different (9).

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ANTIHISTAMINICS AND APOMORPHINE-INDUCED VOMITING[1]

By Eldon M. Boyd and Carl E. Boyd

Abstract

The experiments reported were undertaken to investigate pharmacologically certain claims that some antihistaminics are antemetic in man and capable of inhibiting or preventing apomorphine-induced vomiting in animals. Diphenhydramine hydrochloride, diphenhydramine-8-chlorotheophyllinate, and diphenhydramine-8-bromotheophyllinate, administered to cats in rotation, by mouth, in doses of from 0.5 to 20 mgm. per kgm. body weight 0.5 hr. before subcutaneous injection of 20 to 50 mgm. per kgm. of apomorphine hydrochloride, had no effect upon the vomiting syndrome. Doses of from 0.5 to 40 mgm. per kgm. of the same three derivatives of diphenhydramine were administered orally to dogs in rotation and at intervals of 0.5, 1, 2, and 4 hr. before intramuscular injection of 0.05 mgm. per kgm. of apomorphine hydrochloride, with no effect upon the incidence of vomiting and no effect upon the frequency of vomiting and retching except partial inhibition at convulsant and subconvulsant doses. Similar results were obtained from administration of promethazine hydrochloride orally to dogs in doses of from 0.5 to 40 mgm. per kgm. one hour before intramuscular injection of 0.05 mgm. per kgm. of apomorphine hydrochloride, and the same procedure, substituting methapyrilene hydrochloride and methapyrilene-8-chlorotheophyllinate for promethazine hydrochloride, did not affect the vomiting syndrome whatsoever. The results indicate that in amounts corresponding to usual human therapeutic doses, none of these antihistaminics has any ability to prevent apomorphine-induced emesis in dogs and cats.

Introduction

Attention to the possible antemetic value of the antihistaminics was drawn by reports of several American investigators in 1949 (3, 16, 27) in which the drug employed was diphenhydramine-8-chlorotheophyllinate. After three years of use, the opinion of American investigators is summarized in the conclusion (12) that diphenhydramine-8-chlorotheophyllinate has a temporary preventive and therapeutic value of unknown mechanism in motion sickness. American medical literature contains numerous articles upon the apparently beneficial value of this drug against vomiting in such conditions as radiation sickness (3), pregnancy (9), labyrinthine fenestration reactions (8), and morphine emesis (24). British medical literature contains several references to the apparently successful use of antihistaminics, such as promethazine hydrochloride and pyrilamine maleate, against the vomiting of seasickness and pregnancy (1, 13, 17). Chinn and Oberst (11) reported that the antemetic value of diphenhydramine-8-chlorotheophyllinate was due entirely to its content of diphenhydramine, a conclusion which has been both confirmed and repudiated in subsequent reports (10, 22). In therapeutic trials controlled by use of placebos, considerable doubt has been cast upon the reputed antemetic value of diphenhydramine-8-chlorotheophyllinate in hyperemesis gravidarum (2).

The ability of diphenhydramine-8-chlorotheophyllinate and diphenhydramine hydrochloride to prevent morphine- or apomorphine-induced

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vomiting in dogs has been investigated in several laboratories. Two groups of investigators (15, 25) reported that in doses equivalent to the human therapeutic dose, neither drug inhibited vomiting. Three groups (10, 14, 23) reported that in doses of 20 and 40 mgm. per kgm. body weight there was some decrease in the number of vomiting spells. One group (29) reported complete inhibition of vomiting. One group (22) reported some inhibition of vomiting in cats; another group (25) found no inhibition of vomiting in cats.

Method

In our laboratories at Queen's University, 48 cats were used to determine whether the antihistaminics diphenhydramine hydrochloride, diphenhydramine-8-chlorotheophyllinate, and diphenhydramine-8-bromotheophyllinate could inhibit or prevent apomorphine-induced vomiting. Each antihistaminic was administered by mouth in capsule form, in doses of from 0.5 to 20 mgm. per kgm. body weight, each dose of each drug being given to 6 to 10 cats which in rotation served as controls; 30 min. later a challenging emetic dose of from 20 to 50 mgm. per kgm. of apomorphine hydrochloride was injected subcutaneously. The apomorphine-induced vomiting syndrome in the cat was found to be uninfluenced by previous administration of any dose of any of these antihistaminics. The vomiting syndrome in the cat is accompanied by considerable excitation of the central nervous system owing to the large dose of apomorphine required to produce vomiting in this species which is resistant to the emetic action of the drug (26); further studies were confined to use of dogs.

The three derivatives of diphenhydramine noted above were tested for antemetic activity by oral administration to dogs in capsule form in doses of from 0.5 to 40 mgm. per kgm. body weight and at intervals of from 0.5 to 4 hr. before intramuscular injection of a challenging dose of 0.05 mgm. per kgm. body weight of apomorphine hydrochloride. Each dose of each antihistaminic at each interval was given to 6 to 8 dogs (21 dogs in all were used) of both sexes and 5 to 13 kgm. body weight and the dogs were used in rotation as controls given a placebo capsule. Three other antihistaminics, promethazine hydrochloride (20), methapyrilene hydrochloride, and methapyrilene-8-chlorotheophyllinate were administered in the same doses one hour before injection of apomorphine. Following administration of the emetic agent, there were recorded: the interval in minutes before onset of vomiting, the number of vomiting and retching spells, and a quantitation of physical activity and salivation. It will suffice to report in tabular form data upon the frequency of vomiting spells and a summary of this information is presented in Table I.

Results

Diphenhydramine hydrochloride administration did not prevent vomiting, nor lower the incidence of vomiting, which averaged 92·5%, nor affect the interval before the onset of vomiting, which averaged 7.8 ± 2.2 min. In

TABLE I

THE EFFECT OF ORAL ADMINISTRATION OF ANTIHISTAMINIC DRUGS UPON APOMORPHINE-INDUCED VOMITING IN DOGS

Antihistaminic drug	Mean number of vomiting attacks per dog per dose of antihistaminic drug (mgm. per kgm. body weight)						
	0.5	1.0	2.0	5.0	10.0	20.0	40.0
Administered 0.5 hr. before apomorphine							
Control: no antihistaminic drug	3.1	4.0	4.5	2.8	4.0	5.0	4.3
Diphenhydramine hydrochloride	3.3	3.5	3.8	3.2	3.2	3.3	2.5
Diphenhydramine-8-chlorotheophyllinate	2.2	3.1	3.7	3.0	3.5	3.5	4.0
Diphenhydramine-8-bromotheophyllinate	3.5	2.3	2.8	3.2	5.3	6.0	4.8
Administered 1 hr. before apomorphine							
Control: no antihistaminic drug	3.8	2.9	3.5	4.3	4.2	3.8	5.1
Diphenhydramine hydrochloride	3.1	2.7	3.3	2.8	1.8	2.5	3.5
Diphenhydramine-8-chlorotheophyllinate	3.5	3.3	2.3	4.0	4.0	3.5	4.6
Diphenhydramine-8-bromotheophyllinate	3.0	4.0	2.8	5.3	3.2	3.2	4.0
Administered 1 hr. before apomorphine							
Control: no antihistaminic drug	3.3	4.2	5.3	5.6	4.4	3.7	3.3
Promethazine hydrochloride	4.2	4.0	7.4	6.7	5.5	1.2	2.5
Methapyrilene hydrochloride	3.2	6.5	4.5	5.0	3.4	2.8	Convulsions
Methapyrilene-8-chlorotheophyllinate	3.0	2.5	6.0	5.6	4.4	2.2	Convulsions
Administered 2 hr. before apomorphine							
Control: no antihistaminic drug	3.1	3.9	3.2	3.5	3.9	3.1	3.5
Diphenhydramine hydrochloride	3.0	3.9	3.0	3.5	2.4	1.1	0.7
Diphenhydramine-8-chlorotheophyllinate	2.5	3.5	3.8	5.6	3.3	1.5	3.0
Diphenhydramine-8-bromotheophyllinate	3.0	2.9	3.5	3.7	3.1	2.3	1.2
Administered 4 hr. before apomorphine							
Control: No antihistaminic drug	3.0	2.8	3.0	3.2	3.7	3.2	3.2
Diphenhydramine hydrochloride	3.0	3.7	3.2	3.0	3.2	2.3	Convulsions
Diphenhydramine-8-chlorotheophyllinate	2.3	3.0	3.3	3.7	3.0	3.5	2.0
Diphenhydramine-8-bromotheophyllinate	3.0	2.0	2.7	4.5	3.7	2.7	1.5

doses of 0.5 to 5 mgm. per kgm. it did not affect the frequency of vomiting; in doses of 10 to 40 mgm. per kgm., some of the values listed in Table I were calculated to be significantly ($P < 0.05$) lower than those of the controls, after the criteria of Bradford Hill (21). The frequency of retching, which averaged 27.1 ± 5.2 retching spells in the controls, was also appreciably lowered only by these higher doses of diphenhydramine hydrochloride, which did not significantly affect the excessive salivation of the vomiting syndrome at any

dosage. The higher doses produced a significant increase in physical activity culminating in occasional convulsions at doses of 40 and 60 mgm. per kgm.

Diphenhydramine-8-chlorotheophyllinate likewise did not prevent vomiting, prolong its onset, lessen its incidence, nor affect excessive salivation. The occasional decline in the number of vomiting and retching spells seen after large doses of diphenhydramine hydrochloride was less marked after diphenhydramine-8-chlorotheophyllinate which caused significant excitement in a dose of 40 mgm., and convulsions occasionally at 60 mgm., per kgm. body weight.

The 8-bromotheophyllinate derivative of diphenhydramine was found to have no effect upon the vomiting syndrome, apart from occasional inhibition of the frequency of vomiting and retching in excitant doses.

Studies upon promethazine hydrochloride failed to demonstrate any antemetic value except some partial inhibition of frequency at high dosage levels (Table I). In no dose up to the convulsant level of 40 mgm. per kgm. did either of the two derivatives of methapyrilene exhibit any significant antemetic activity (Table I).

Discussion

The experiments described above demonstrated that none of the antihistaminics used was capable of preventing vomiting in cats or dogs following injection of apomorphine hydrochloride. The antihistaminics were administered in amounts covering doses from one corresponding to less than the human therapeutic dose, on a body weight basis, up to doses which produced toxic effects. These doses were administered orally to dogs at intervals of from 0.5 to 4 hr. before injection of apomorphine hydrochloride. The antihistaminics were not injected subcutaneously nor intramuscularly because in many instances, particularly with higher doses of diphenhydramine (6, 18), this route of administration leads to local induration and ulcer formation. Intravenous administration to dogs of diphenhydramine hydrochloride in doses of 1 and 2.5 mgm. per kgm. and of diphenhydramine-8-chlorotheophyllinate in doses of 2 and 5 mgm. per kgm. has been reported to have no effect upon the incidence and character of vomiting following intravenous injection of a minimal emetic dose of apomorphine hydrochloride 15 min. later (25).

The use of apomorphine hydrochloride as a challenging agent should expose the antemetic activity of any drug acting upon or distal to the vomiting center. More precisely, apomorphine hydrochloride has been found to stimulate vomiting by acting upon an emetic trigger zone situated in the dorsolateral portion of the ala cinerea in the floor of the fourth ventricle of the dog (5, 28) and probably between the ala cinerea and the vestibular complex in the cat (4). Should antihistaminics act as antemetics upon the labyrinth and vestibular nerve, as has been proposed for diphenhydramine-8-chlorotheophyllinate (19), use of apomorphine hydrochloride as a challenging agent could expose no antemetic activity.

Intramuscular injection of apomorphine hydrochloride in a dose of 0.05 mgm. per kgm. is a powerful emetic stimulus in the dog. As noted above, almost all dogs vomited after receiving this injection. Previous oral administration of phenobarbital in a dose of 30 mgm. per kgm., or of atropine sulphate or hyoscine in a dose of 1 mgm. per kgm., does not affect the vomiting syndrome (10). While it might be considered that no measure short of general anaesthesia could prevent this apomorphine-induced vomiting, the syndrome has been found to be prevented completely by previous administration of doses of the compound (dimethylamino-1-*n*-propyl-3)-*N*-(2-chloro)-phenothiazine hydrochloride) in doses which cause little or no sedation of physical activity in the dog (7).

White *et al.* (29) reported that 1 mgm. per kgm. of diphenhydramine hydrochloride and 2 mgm. per kgm. of diphenhydramine-8-chlorotheophyllinate administered orally before subcutaneous injection of 30 mgm per kgm. of apomorphine hydrochloride into dogs produced complete inhibition of vomiting. In a reprint sent to one of us, the "30 mgm." was corrected to read "30 microgm.". To test the possibility that the antihistaminics might be antemetic toward a dose of apomorphine less provocative of vomiting than the 0.05 mgm. per kgm. used above, (this dose, or larger doses, of apomorphine has been commonly used by other investigators (10, 14, 22, 23)), six dogs were given in rotation 0.05, 0.02, and 0.01 mgm. per kgm. of apomorphine hydrochloride one hour after oral administration of diphenhydramine-8-chlorotheophyllinate in a dose of 20 mgm. per kgm. The antihistaminic had no antemetic action against the smaller doses of apomorphine which produced average frequencies of vomiting of 1.3 and 0.2 respectively.

The results confirm previous reports (10, 14, 23) that the derivatives of diphenhydramine may partially inhibit apomorphine-induced vomiting in toxic or near-toxic doses and add the antihistaminic promethazine hydrochloride to this group. They also explain the failure of others (15, 25) to obtain any inhibition of apomorphine-induced vomiting as due to use of lower doses of diphenhydramine derivatives.

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A COMPARISON OF THE METHOD OF FLAME PHOTOMETRY WITH THE CHEMICAL METHODS FOR THE DETERMINATION OF CATIONS IN TISSUES[1]

By J. S. Barlow and J. F. Manery

Abstract

A procedure has been described whereby the Perkin-Elmer flame photometer, Model 52A, can be used to analyze 10 ml. of a solution of ashed biological materials, the solution containing 0.5 m.e. of sodium or potassium per liter. The lithium concentration used for the internal standard, the optimal rate of flow through the atomizer, the air and gas pressures, and cation interferences are discussed. Applying the modifications and precautions adopted in this laboratory the results of photometer analyses of tissues and serum were shown to compare favorably with those obtained from well-established chemical methods. The merits and demerits of both procedures are outlined.

Introduction

In a recent publication Hald (3) has outlined the scope of flame photometry; she has discussed the degree of accuracy which can be expected and the general technical procedures involved, as well as various modifications of the instrument. As Hald points out the instrument should be set up by someone trained in quantitative analytical chemistry, and closely supervised instruction and practice is required before an operator can be expected to obtain acceptable results.

This present publication is being made because of the many conflicting opinions concerning the general usefulness of the instrument, the individual difficulties of various investigators which are not published, and because of the dearth of data demonstrating that the instrument has been properly tested. It is our opinion that the most reliable tests are made in laboratories in which accurate chemical methods for the determination of the cations have been well established. Because these chemical methods are time-consuming, tedious, and difficult, they are not in general use in many places. The Perkin-Elmer flame photometer, Model 52A, has been used in this laboratory for the past five years and its performance has been compared to chemical methods already well established. Specific difficulties encountered in the operation of this instrument will be described.

Methods

Power Supply

A motor generator was used throughout this research to supply alternating current of the desired frequency. How much of the instability of the apparatus can be attributed to such a power supply cannot be assessed at this time.

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Contribution from the Department of Biochemistry, University of Toronto, Toronto, Ontario.

Air and Gas Pressure

For this particular model it was imperative that the air and gas pressure be maintained constant while the instrument was in use, although an accurate setting at exactly 10 lb. for air and 2–3 lb. for acetylene or propane was not essential. Two-stage regulators were necessary in both lines and the pressure gauges provided on these regulators were not sensitive enough to show the small pressure changes which had a profound effect on the instrument. Mercury manometers were installed in the lines in order to observe small changes in pressure.

Operation of Atomizer and Optimal Rate of Flow

The rate of flow of solution through the atomizer was easily controlled by a needle valve. In order to conserve material, the flow was reduced as far as was practical. Reducing the rate from approximately 20 ml. per min. (atomizer wide open) to 2 ml. per min. decreased the galvanometer deflection by only 20%. Further reduction of flow did not appear to be advisable, for it caused a disproportionately large decrease in galvanometer deflection and the small orifice remaining in the atomizer became very susceptible to clogging.

It was impossible to avoid occasional clogging of the atomizer at this setting even when the solutions had been filtered (see section on sample preparation); the clog could be easily removed by opening the needle valve fully, a procedure which destroyed the setting. In order to return to the original setting a dial was attached to the body of the atomizer and a pointer to the shaft of the needle valve. The initial setting determined by experiment was thereafter noted by the position of the pointer on the dial and, when the needle valve was opened to unclog the atomizer, it could be readily closed again to the initial setting.

The Optimal Concentrations of Cations

The upper limit of the concentration range in which the determinations are to be made should produce a galvanometer deflection of at least 50 scale divisions. If, in order to obtain this deflection, it is necessary to increase the amplification to a value close to the maximum, it will be found that the galvanometer becomes so sensitive to external influences that accurate readings are difficult to obtain. The advantage gained by using a low concentration will be lost owing to the inaccuracy of the reading. In our experience, a concentration of 1 m.e. per liter was found to be satisfactory for the upper limit of both sodium and potassium determinations, and this concentration will, therefore, be used to represent this limit in the remainder of the discussion. With the upper limit of the concentration range set at 1.0 m.e. per liter, the average concentration of the unknowns was about 0.6 m.e. per liter. Since 10 ml. of solution were required for the triplicate readings, each determination required 0.006 m.e. of sodium or potassium. Six milliequivalents of calcium per liter, and over 20 m.e. of magnesium per liter were required to

give this minimal deflection with suitable amplification, and therefore the instrument was considered to be insufficiently sensitive to these elements for our purposes.

The concentration of lithium to be used as the internal standard should be of such a magnitude that, when the current produced by the internal standard photocell is fully amplified (internal standard dial set at 100), it will exactly balance the current output from the photocell which is measuring the 1.0 m.e. of sodium or potassium per liter. If the concentration of lithium is too high, the current produced by the light from the sodium or potassium will have to be amplified to such an extent that, as explained above, the galvanometer will be unsteady. If the concentration of lithium is low, on the other hand, the reduction of amplification of the current produced by light from sodium or potassium will reduce the sensitivity of the instrument. A lithium concentration of 30 m.e. per liter has been found to be satisfactory for potassium analyses, while sodium analyses require only 3 m.e. of lithium per liter.

Calibration Curves and Cation Interference

Since the ratio of the intensity of the light emitted by sodium or potassium to the intensity of the light emitted by the lithium in the flame does not bear a straight line relationship to the concentration of sodium or potassium in the solution, a curve must be drawn to calibrate the instrument. This is done using the rate of flow, concentration of lithium, and the concentration range previously determined. In this laboratory, the calibration curves were drawn by introducing standard solutions containing 1.0, 0.8, 0.6, 0.4, and 0.2 m.e. of sodium or potassium per liter into the flame and adjusting the internal standard dial to maintain the galvanometer at its original setting (50). For potassium determinations, all the standard solutions contained 30 m.e. of lithium per liter; for sodium determinations all the solutions contained 3 m.e. of lithium per liter. The readings taken from the internal standard dial were plotted against the known concentration of sodium or potassium in the solutions. These curves were reproducible within 2%.

Interference is not entirely absent even when an internal standard is used. Since the instrument is far less sensitive to calcium and to magnesium than to sodium and potassium, and since calcium and magnesium are generally present in low concentrations in most animal tissues, they do not present a problem. Sodium and potassium, are often present in relatively high concentrations in animal tissues. Moreover, some materials such as serum contain a comparatively high concentration of sodium relative to the potassium concentration (approximately 23 : 1 in the serum of the chicken) while others, such as muscle, contain a comparatively high concentration of potassium compared to sodium (approximately 5.5 : 1 in the muscle of the adult chicken). It was found that sodium interfered considerably with potassium analyses. With a sodium : potassium ratio of 1 : 1 there was no increase in the reading obtained on the solution containing 0.8 m.e. per liter; the reading obtained on the solution containing 0.2 m.e. per liter was increased by 2%. When the ratio

TABLE I

COMPARISON OF CHEMICAL AND FLAME PHOTOMETER METHODS FOR THE DETERMINATION OF Na, K, Ca, AND Mg

Type of analysis		Method of analysis	
		Chemical	Flame photometer
K	Amount required	0.005 m.e.	0.005 m.e.
	Tissue equivalent		
	Serum	1 ml.	1 ml.
	Muscle	0.05 gm.	0.05 gm.
	Accuracy		
	Difference between duplicates	2.0% (12)	2.2% (5)
Na	Amount required	0.05 m.e.	0.005 m.e.
	Tissue equivalent		
	Serum	0.4 ml.	0.033 ml.
	Muscle	3.0 gm.	0.25 gm.
	Accuracy		
	Difference between duplicates	1.6% (5)	5.6% (7)
Ca	Amount required	0.01 m.e. or less	0.025 m.e.
	Tissue equivalent		
	Serum	4 ml.	10 ml.
	Muscle	4 gm.	10 gm.
Mg	Amount required	0.0025 m.e.	0.2 m.e.
	Tissue equivalent		
	Serum	2.5 ml.	200 ml.
	Muscle	0.012 gm.	10 gm.

NOTE: *Numbers in parentheses represent the number of duplicates on which the averages are based.*

of sodium : potassium was increased to 50 : 1, the results were 6% high on the solution having a potassium concentration of 0.8 m.e. per liter, and 12% high on the solution containing 0.2 m.e. per liter. Similarly potassium was found to interfere, though to a lesser degree, with the sodium determinations. In order to make allowance for these interferences, standard solutions having approximately the same Na : K ratio as the unknown were used. These solutions were called working standards to distinguish them from the pure standards used to prepare the calibration curve. The working standard was compared with the pure standard of the same concentration. The unknown solution was then compared with the working standard, and the ratio of the reading given by the working standard to that given by the pure standard of the same concentration was used to determine the corrected reading of the unknown solution.

Preparation of Samples for Analysis

The tissues were routinely ashed at a temperature between 500 and 550° C., the ash dissolved in *N* hydrochloric acid and transferred to a volumetric flask of such a size that the concentration of the element to be determined lay in the working range (1.0 to 0.2 m.e. per liter). An amount of lithium was added to the flask such that the final concentration would be 30 m.e. per liter for potassium analyses and 3 m.e. per liter for sodium analyses and the flask was filled to the mark. Only pyrex glass was used throughout all of the procedures.

To avoid repeated clogging of the atomizer it was necessary to filter solutions of tissue ash. Filter paper could not be used because even the finest grades added appreciable quantities of sodium to the solution. Hence all solutions were filtered with gentle suction through sintered glass filters of medium porosity.

Performance

Applying the refinements and precautions just outlined, the method of flame photometry for cation determinations was compared with chemical methods with regard to the accuracy, the quantity of material required, and the time necessary to carry out the analyses. The data are summarized in Table I. All the figures reported for flame photometry are results of triplicate analyses. The triplicate analyses generally had a range of about 3% and amounts of material required are sufficient to permit three determinations to be made.

TABLE II

RECOVERIES OF K FROM ASHED RAT MUSCLE

Wt. of muscle, gm.*	Absolute amount of K (m.e.)			% Error**	K per kgm. muscle (m.e.)	Range between duplicates, %
	Added	Found	Calc.			
4.719		0.522			110.7	5.3
3.550		0.416			116.7	
1.614	0.200	0.388	0.384	+1.0		
1.815	0.200	0.416	0.406	+2.5		
3.236		0.388			119.9	5.3
4.110		0.459			111.9	
1.932	0.200	0.440	0.424	+4.5		
1.585	0.200	0.403	0.384	+5.0		
3.995		0.446			113.0	1.9
3.728		0.430			115.2	
1.826	0.200	0.406	0.408	−0.5		
1.849	0.200	0.420	0.411	+2.2		

* *1/50 of sample used to make 25 ml. of solution for analysis.*

** $\%\ Error = \frac{K\ found - K\ calc.}{K\ calc.} \times 100.$

It is in the determination of sodium that the instrument has proved to be of greatest value. The chemical analyses, with which the results obtained from the flame photometer were compared, were carried out by a semimicro modification of the technique of Butler and Tuthill (2), which was described previously in detail (1). The chemical method will measure 0.05 m.e. of sodium with an accuracy of $\pm 2\%$; however, it requires precipitation of phosphates from the sample prior to the analysis, a procedure which causes the loss of about one-fifth of the sample. Hence the original piece of tissue should contain about 0.06 m.e. of sodium; the optimal amount of muscle required for sodium determination by this chemical method is about 3 gm., and 0.4 ml. is the optimal amount of plasma required.

The flame photometer, on the other hand, will measure 0.005 m.e. of sodium with an average difference of 5.6% between duplicates in a typical series of seven sets of duplicates. In many researches the decrease in accuracy was greatly overweighed by the reduction in the amount of material required since the amount of tissue required for analysis, 0.033 ml. of blood plasma or 0.25 gm. of muscle, is only 1/12 of the amount required for analysis by the chemical method.

In the determination of potassium the flame photometer does not permit a similar saving in the amount of tissue required. The semimicro modification of the technique of Shohl and Bennett (4), in use in this laboratory (6), will determine about 0.005 m.e. of potassium with an average difference between duplicates of 2.0%. This is the optimum amount of potassium which can be determined with the flame photometer by the procedure described here. Since no separation of interfering ions is necessary in either method each will require the same quantity of material for analysis, about 1 ml. of plasma or 0.05 gm. of muscle.

A preliminary examination of the ability of the flame photometer showed that 0.025 m.e. of calcium would be the minimum amount required for analysis. Since approximately 5 ml. of plasma or 10 gm. of muscle would be required to supply this amount of calcium, the procedure was not considered to be satisfactory for the study which was being carried out. Similarly, 0.2 m.e. of magnesium would be required for analysis using the flame photometer. This is contained in about 10 gm. of muscle and about 200 ml. of plasma.

In discussing the performance of the flame photometer it is important to consider the time required to perform the analyses, as well as the amount of material required and the accuracy of the method. Performing the triplicate analyses which were required to obtain the stated degree of accuracy with the flame photometer, not more than 24 samples were analyzed in one day (eight continuous hours), since as much as two hours or more was often spent in getting the instrument stabilized and standardized at these low concentrations. This did not represent a saving of time in potassium analyses. A competent assistant such as Miss E. J. Murphy, to whom we are indebted for the chemical analyses, can perform as many, if not more, potassium analyses by the chemical method in eight working hours, although the samples were allowed to stand

overnight at one point in the procedure. In her opinion the chemical method was more consistently reliable and satisfying. The chemical method which was used for sodium analyses on the other hand, was a gravimetric method and with the removal of phosphates which has been mentioned above, required considerable time for precipitation, filtering, and re-aliquoting. Not more than 12 samples could be carried through this entire procedure in an eight-hour day, so that the flame photometer effected a 100% saving in time in the performance of sodium analyses.

Still another factor of importance when discussing methods of analysis is the time required for the operator to become proficient in the technique. After the flame photometer has been set up, and the methods established by a competent chemist, reasonably accurate results can be obtained by an operator with as little as two days of closely supervised instruction. The technique, once learned, can be applied to any cation. At least two days of closely supervised instruction, and perhaps longer, would be required for

TABLE III

RECOVERIES OF Na FROM ASHED RAT MUSCLE

Wt. of muscle, gm.*	Absolute amount of Na (m.e.)			% Error**	Na per kgm. muscle (m.e.)	Range, %
	Added	Found	Calc.			
4.719		0.0970			20.5	11.6
3.550		0.0819			23.1	
1.614	0.0300	0.0685	0.0652	4.4		
1.815	0.0300	0.0749	0.0696	6.4		
3.236		0.0855			26.5	13.3
4.110		0.0950			23.2	
1.932	0.0300	0.0808	0.0779	3.6		
1.585	0.0300	0.0687	0.0693	0.9		
0.9820		0.0219			22.6	
0.9882		0.0220			22.6	9.1
0.9608		0.0216			22.4	
0.9796		0.0202			20.6	
0.4784	0.01	0.0211	0.0208	+1.5		
0.5050	0.01	0.0212	0.0212	0		
0.5025	0.01	0.0206	0.0212	−3.0		
0.4969	0.01	0.0196	0.0202	−3.0		
	0.02	0.0200		0		
	0.02	0.0196		−2.0		
	0.02	0.0200		0		3.0
	0.02	0.0202		+1.0		

* *1/25 of sample used to make 10 ml. of solution for analysis.*

** $\%\ Error = \frac{Na\ found - Na\ calc.}{Na\ calc.} \times 100.$

each of the chemical techniques. This would therefore be a factor of considerable importance in a laboratory in which people with experience in the chemical methods were not available.

In summary then, it may be said that the flame photometer will reduce to 1/12 the amount of material, and to 1/2 the amount of time required for sodium analyses without unduly increasing the error of the determination. A second advantage lies in the fact that the technique, once learned, is applicable to all cations. Calcium and magnesium determinations can probably be made more efficiently by chemical methods unless large amounts of these elements are present; 0.025 m.e. of calcium and 0.2 m.e. of magnesium are the minimum amounts which could be satisfactorily determined on the flame photometer.

Analyses of Biological Materials

The accuracy of the flame photometer, when operated as outlined above, was tested by determining its ability to recover added amounts of sodium and potassium from rat muscle and serum, and by comparing the results of flame photometer analyses to those obtained by the well-established chemical procedures. In all experiments using solid tissues a fine mince was made before sampling.

The results are presented in Tables I, II, III, and IV. In preparing these summaries of the results, the percentage error was calculated on the total concentration of the element in the sample rather than on the amount added. For this reason the sodium and potassium contents of the muscle and serum, as determined from the duplicate analyses, were averaged to determine the amount of the element in the tissue. The amount of sodium or potassium in the tissue, as calculated from the average value, plus the amount of the element added gave the calculated amount of sodium or potassium in the sample. The differences between duplicate analyses are given in the columns labelled "Range" as a percentage of the average of the duplicates.

From Table II it can be seen that the range between duplicate potassium analyses on rat muscle is about 5%. It is probable that some of this difference is due to the difficulties of obtaining a uniform sample from the muscle mince, since the range between duplicate analyses carried out on serum is generally less (3%). The recoveries of added potassium from muscle vary from 5% high to 0.5% low. It would thus appear that the average error in potassium determinations made with the flame photometer is less than 5%. Indeed, in Table I which has been presented earlier to summarize the relative merits of the flame photometer and chemical procedures, the average difference between duplicates in a series of five pairs of measurements was 2.2%.

The range between duplicate analyses of rat muscle for sodium, presented in Table III, is about 12% and the quadruplicate analyses all lie within a 9% range. As has been pointed out above, a portion of this difference is probably due to error in sampling the muscle because the range between duplicate analyses of serum (Table IV) or quadruplicate analyses of standard solutions

TABLE IV

RECOVERIES OF K AND NA FROM ASHED RAT SERUM

Vol of serum, ml.*	Absolute amount (m.e.)			% Error**	Conc./liter serum (m.e.)	Range between duplicates, %
	Added	Found	Calc.			
Potassium						
4		0.0195			4.62	0
4		0.0195			4.62	
2	0.01	0.0207	0.0198	+4.5		
2	0.01	0.0205	0.0198	+3.5		
4		0.0225			5.55	2.8
4		0.0231			5.71	
2	0.01	0.0209	0.0214	−2.5		
2	0.01	0.0208	0.0214	−3.0		
Sodium						
4		0.560			140.0	0.6
4		0.562			140.6	
2	0.300	0.576	0.581	0.9		
2	0.300	0.583	0.581	0.3		
4		0.588			145.0	1.7
4		0.600			147.8	
2	0.300	0.602	0.597	0.8		
2	0.300	0.601	0.597	0.8		

* *1/25 of sample used to make 50 ml. of solution for analysis.*

** $\%\ Error = \frac{K\ (or\ Na)\ found - K\ (or\ Na)\ calc.}{K\ (or\ Na)\ calc.} \times 100.$

(Table III) is much less. The maximum error in sodium recoveries from muscle is 6.4% and the average error is less than 3%. The errors in recoveries of sodium added to serum are less than 1%.

The results of chemical and photometric determinations of the sodium and potassium concentrations in the tissues of the chicken are illustrated in Table V. In this test both methods of analysis were performed on the same solution of the ash. For this reason a comparison of the results obtained using the photometer with the results obtained using the chemical methods will not be subject to errors in sampling. The differences between the results of the two sodium methods, expressed as a percentage of the results of the chemical analyses, range from 0 to −7%. The average difference is −3.4% and the root mean square deviation from the average is 1.8%. The differences between the results of the two potassium methods range from +1% to −4% of the result obtained by the chemical procedure. The average difference is −1.2% of the chemical determination and the root mean square deviation from the average is 2%.

TABLE V

COMPARISON OF CHEMICAL AND FLAME PHOTOMETRIC ANALYSES OF ADULT CHICKEN TISSUES

Tissue	Na,m.e./kgm.			K,m.e./kgm.		
	Chem.	Photom.	Diff. as % of chemical	Chem.	Photom.	Diff. as % of chemical
Breast muscle	24.6	23.8	−3	93.3	94.5	1
	24.8	24.2	−2	95.8	96.5	1
Leg muscle	33.6	32.1	−4	95.2	92.1	−3
	37.4	35.9	−4	92.8	93.8	1
Heart muscle	59.1	56.9	−4			
Gizzard	34.6	33.4	−4	105.9	102.5	−3
	35.0	33.6	−4	102.9	100.8	−2
Liver	62.5	60.8	−4			
	57.8	57.3	−1			
Tendon	101.3	94.5	−7			
Serum	163.2	161.2	−1	6.8	6.75	−1
	161.0	161.2	0	6.9	6.66	−4
Average difference			−3.2			−1.2

When the flame photometer is used to perform potassium analyses, the range between analyses of duplicate samples is in the order of 5%, the method will recover added potassium within 5% and agrees with the well-established chemical method within $-1.2 \pm 2\%$; when used to perform sodium analyses, the range between analyses of replicate samples of tissue is in the order of 12%, added sodium is recovered within 6.4%, and the results agree with results obtained by the chemical method within $-3.2 \pm 1.8\%$. It is our practice to use the chemical methods whenever small differences have to be determined with high accuracy.

These results might be compared with a number of analyses performed with a similar instrument by Steinbach (5). Steinbach's analyses were performed on whole muscle homogenates containing about 0.08 gm. of muscle per ml. The homogenates were divided into four portions from which 1 ml. and 2 ml. samples were taken for analyses; thus eight determinations were performed on each sample. These samples of homogenates were digested with nitric acid, diluted to 15 ml. volume, centrifuged if necessary to remove debris, and analyzed with a Perkin Elmer Model 52A flame photometer. A calculation of the approximate concentration of sodium and potassium in the final solutions used for analysis indicates that the solutions containing 1 ml. of the homogenate contained about 0.2 and 0.3 m.e. of sodium and potassium respectively per liter, and the solutions containing 2 ml. of the homogenate contained 0.4 and 0.6 m.e. of sodium and potassium respectively per liter. The solutions

containing 1 ml. samples of the homogenate were therefore slightly more dilute than the samples which have been used in this study (0.5 m.e. of sodium or potassium per liter) while the samples containing 2 ml. of the homogenate were of approximately the same concentration. Steinbach used the flame photometer as a direct reading instrument with no internal standard.

Steinbach's results are presented, not as the range between the determinations as has been done in Tables II, III, and IV, but as deviations of the analyses from their mean. These values would therefore be approximately one-half the magnitude of the range which has been expressed in the tables presented here. The maximum deviation which Steinbach reports in a series of 16 sodium analyses is 15%, compared to a maximum range of 12% in the internal standard method reported here. In a similar series of potassium analyses, Steinbach presents a maximum deviation of 25%, as compared to a maximum range of 5% obtained by the method reported here.

Summary

1. A procedure has been outlined whereby the Perkin-Elmer Model 52A flame photometer may be used to analyze solutions of ashed biological materials containing approximately 0.5 m.e. of sodium or potassium per liter with a range not greater than 5% for potassium analyses and not greater than 12% for sodium analyses. The results of the analyses performed by this procedure have been found to agree with the results of analyses performed by well-established chemical methods within $-1.2 \pm 2\%$ for potassium analyses and with $-3.2 \pm 1.8\%$ for sodium analyses.

2. Since 10 ml. of final solution were required to perform the analyses with the above degree of accuracy, the determinations were actually carried out on about 0.5 μe. of sodium and potassium. This quantity of sodium is contained in about 0.033 ml. of serum or about 0.25 gm. of muscle. The same quantity of potassium corresponds to 1 ml. of serum or 0.05 gm. of muscle. The chemical sodium method requires at least 10 times this amount, therefore sodium analyses can be performed with the flame photometer on much less tissue than can chemical sodium analyses. The chemical potassium method requires no more material than the flame photometer potassium method and is used routinely when highest accuracy is required.

3. When operating at maximum efficiency, the flame photometer consumes 2 ml. of the solution being analyzed per minute. At this rate of flow, a preliminary reading to determine the approximate concentration of the unknown element, and three accurate readings in which the unknown is compared to a standard of similar composition can be made on 10 ml. of solution.

4. When choosing the range of concentrations in which the flame photometer is to be operated, the upper limit of the range should be sufficient to cause the galvanometer to deflect 50 scale divisions without undue amplification which leads to instability.

5. The concentration of lithium chosen as the internal standard should be such that, at full amplification, the current which it produces will balance the current produced by the concentration of the unknown element which is chosen as the upper limit of the working range.

6. It was found necessary to control the pressures of air and gas very carefully. In order to detect variations in these pressures, mercury manometers were put in the gas and air lines.

7. A dial and indicator installed on the atomizer needle valve so that it can be easily returned to a previously established setting has been found to be a worthwhile modification.

8. Clogging of the atomizer can be largely avoided by filtering the air supplied to the atomizer and by filtering the solutions before analysis through sintered glass filters of medium porosity.

9. A high concentration of sodium in the solution has been found to interfere seriously with the potassium analyses causing an error of +12% in a solution containing 0.2 m.e. of potassium and 10.0 m.e. of sodium per liter. The interference caused by potassium in solutions being analyzed for sodium was much less. A method of circumventing this interference by using standard solutions containing approximately the same sodium : potassium ratio as the unknown has been described.

10. The instrument has been found to be too insensitive to calcium and magnesium to be of value for determination of these elements in most animal tissues.

Acknowledgments

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THE ANTIGENICITY OF CHOLERA VACCINE PREPARED IN FLUID MEDIUM[1]

By L. E. Ranta[2] and Paula M. McCreery[3]

Abstract

Cholera vaccine prepared in a chemically defined fluid medium (containing tyrosine, asparagine, glycine, and inorganic salts) was compared antigenically in mice with vaccines prepared by standard methods. The mean LD_{50} for normal mice was 108 ± 9.1 *V. comma* of the strain employed for challenging. Both vaccines afforded a high degree of protection. Mice immunized with bivalent (Ogawa and Inaba) Standard vaccine had a mean LD_{50} of 40.0 ± 4.7 million *V. comma*. Mice protected by bivalent fluid-prepared vaccine had a mean LD_{50} of 32.7 ± 6.0 million *V. comma*. The difference is not statistically significant. Vaccines prepared in fluid medium were relatively unstable.

In 1950, Ranta and McLeod (7) reported upon a study of the growth of *Vibrio comma* in fluid media of chemically defined compositions. Under appropriate aeration with a mixture of air and carbon dioxide, the growth of *V. comma* developed a turbidity equivalent to a 1600 p.p.m. silica standard in a medium prepared by dissolving in 1 liter of distilled water the following compounds: 0.67 gm. of tyrosine, 0.42 gm. of asparagine, 0.51 gm. of glycine. 5.0 gm. of sodium chloride, 5.0 gm. of ammonium sulphate, 0.75 gm. of dipotassium hydrogen phosphate, 0.1 gm. of magnesium sulphate, 10.0 gm. of glucose, and 15.0 gm. of sodium bicarbonate. The growth obtained in this tyrosine–asparagine–glycine medium (TAG medium) was diluted with physiological saline to produce a final concentration of 8000 million *V. comma* per ml., and the organisms were killed by adding phenol to a final concentration of 0.5%.

In the study to be reported here, TAG vaccines were compared antigenically with cholera vaccines (Standard) produced by the method described by Ranta and Dolman (5) in 1943. Antigenicity tests were carried out by the mouse protection test reported by Ranta and Dolman (6) in 1944, so modified that the number of *V. comma* required to kill 50% of immunized mice (LD_{50}) could be determined.

Immunizing and Challenging Procedures

Groups of white mice were immunized with TAG vaccine containing *V. comma*, Strain No. 41–1 (Ogawa), Strain No. 35–A–3 (Inaba), or equal concentrations of these strains. Monovalent and bivalent vaccines were adjusted turbidimetrically to contain 8000 million *V. comma* per ml. before storage.

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The vaccines were diluted 1 : 5 with physiological saline before intraperitoneal inoculation of the mice. Each animal received dosages of 0.25 ml. and 0.5 ml. spaced a week apart.

Two weeks after the second dose, the vaccinated mice were divided into smaller groups having 6 to 30 mice in the various tests. A series of appropriate dilutions of the challenging Ogawa or Inaba cultures was prepared. Each group of mice was inoculated with differing numbers of vibrios so that LD_{50}'s could be calculated. The final dilutions were made into 5% mucin in physiological saline.

Using the same procedure, groups of white mice were immunized with similar doses of Standard vaccine containing equal concentrations of the Ogawa and Inaba strains adjusted to have 8000 million *V. comma* per ml. Subsequent challenges with mucinized *V. comma* were made to determine the LD_{50}'s of mice protected by the Standard vaccine.

Controls were established by challenging normal white mice with dilutions designed to yield the LD_{50} end point. The challenging strains of *V. comma* were stored in a lyophilized state throughout the experimental work.

The number of organisms in the challenging dosages was determined by appropriate dilutions into physiological saline from the most dilute suspension prepared for the challenge. The dilutions for counting were so selected that an inoculum of 0.5 ml. would yield 100 to 300 colonies in each of four, nutrient agar, poured plates at pH 8.3 after 24 hr. incubation at 37.5° C. The resulting counts permitted calculations of the number of viable *V. comma* in the mucinized suspensions inoculated into the groups of mice.

The challenging doses for immunized animals were prepared by seeding *V. comma*, freshly revived from a lyophilized state, onto proteose–peptone water agar slants (1.0% proteose–peptone, 0.5% sodium chloride, and 2.0% agar dissolved in distilled water and adjusted to pH 8.3 and warmed to 37.5° C. before inoculation). After six hours' incubation at 37.5° C., the organisms were washed off the slants with physiological saline. The suspension was diluted with physiological saline to correspond with the turbidity of a 500 p.p.m. silica standard (1). Several determinations of the number of *V. comma* contained in a suspension equivalent to a 500 p.p.m. silica standard gave a mean value of 2451 $\pm$ 14.1 million *V. comma* per ml. Appropriate dilutions of the standardized suspensions were made with physiological saline, the final dilutions being prepared in 5% mucin, of which 0.5 ml. constituted a challenging dose.

The mucinized dilutions used in determining the LD_{50} of immunized mice were 1 : 5, 1 : 10, 1 : 20, 1 : 30, 1 : 40, and 1 : 50 dilutions of the standardized suspension, with plate counts being carried out on a $1 : 10^7$ dilution in physiological saline. Normal mice were challenged with the following mucinized dilutions of the standardized suspension: $1 : 10^6$, $1 : 5 \times 10^6$, $1 : 10^7$, $1 : 5 \times 10^7$, and $1 : 10^8$. Similarly, plate counts were done on a $1 : 10^7$ dilution in physiological saline.

Comparison of Antigenicity of TAG and Standard Vaccines

Challenging normal mice with intraperitoneal inoculations showed a mean LD_{50} of 108 ± 9.1 *V. comma*, Strain No. 41–1 (Ogawa). Normal mice similarly inoculated with mucinized *V. comma*, Strain No. 35–A–3 (Inaba) showed a mean LD_{50} of 86 ± 16.

A difference was demonstrated in the ability of the Inaba and Ogawa strains to produce protection against one another and against themselves. When mice were immunized with Strain No. 35–A–3 grown in TAG medium and challenged with Strain No. 41–1, the LD_{50} was 39 million *V. comma*, while challenges with Strain No. 35–A–3 showed an LD_{50} of 44 million *V. comma*.

Mice vaccinated with TAG vaccine prepared from Strain No. 41–1 and challenged with Strain No. 41–1, showed an LD_{50} of 59 million *V. comma*, while similarly vaccinated mice met the challenge of an inoculation with Strain No. 35–A–3 with an LD_{50} of 52 million *V. comma*.

These differences, though signifying a greater ability to immunize against challenges by the homologous type, were considered to be of such little practical significance that subsequent challenges were made with Strain No. 41–1. This strain was selected as it had demonstrated its ability to produce sharply readable results in contrast to the irregular incidence of deaths up to 72 hr. following challenge with Strain No. 35–A–3. Moreover, challenging with one strain would not interfere with comparison of the immunizing ability of bivalent TAG and Standard vaccines.

Mice immunized with bivalent TAG vaccine and challenged by Strain No. 41–1 produced LD_{50}'s of 22, 27, 31, 33, 40, and 43 million *V. comma*. Mice immunized with equivalently bivalent Standard vaccine produced LD_{50}'s of 32, 37, 42, and 49 million *V. comma*. The significance of these findings will be discussed later.

Instability of TAG Vaccine

During the preparation and use of TAG vaccine, it was frequently noted that lysis was more rapid and extensive than in Standard vaccine. Sometimes, when early growth in TAG medium was slow, the lysis prevented an appreciable increase in turbidity.

To this lysis was attributed a development that sometimes occurred during immunization with TAG vaccine. It was noted that the number of fatalities of mice being inoculated with TAG vaccine was higher than the fatalities among those receiving Standard vaccine. During one experiment, when 40 mice were being immunized in each of the TAG and Standard groups, 11 mice (27.5%) died in the TAG group, while 5 (12.5%) died in the Standard group. These groups had been kept in separate cages during the three weeks of the immunization period.

In order to test the effects of environment which might have influenced the survival of the animals, 80 mice were housed in the same cage. Half of the mice were immunized with TAG vaccine in the usual manner, while the remainder were marked and received Standard vaccine. Three weeks after immunization was begun, none of the mice immunized with Standard vaccine

had died, while 10 of the TAG-immunized mice has died at irregular periods. Most of the fatalities occurred during the early stages of the immunization. The fatalities could not be attributed to contamination of the vaccine. The animals went through a stage of inanition, then succumbed.

The preponderance of deaths among TAG-immunized mice was not, however, consistent. It was apparently related to the aging of certain TAG vaccines, as the vaccines used soon after preparation produced no significant number of deaths. The impression was gained that TAG vaccine deteriorated in turbidity at a more rapid rate than Standard vaccine, although no turbidimetric values were systematically determined.

Further evidence of instability was afforded by skin tests. TAG and Standard vaccines that had been allowed to age produced comparable results when 0.1 ml. quantities of the vaccines, undiluted and diluted 1 : 2, 1 : 4, and 1 : 8, were inoculated into the skin of a guinea pig. The areas of erythema ranged from 1.3 cm. in the greater concentrations to 0.6 cm. at the inoculation site of the 1 : 8 dilution. In the higher dilutions, the sites where TAG vaccine had been inoculated tended to be more œdematous. Both vaccines produced small (0.2 cm.) areas of necrosis in the sites where the two greater concentrations had been injected. The similarity of these results led to an examination of the fluid portions of freshly prepared vaccines.

Freshly prepared TAG and Standard vaccines were centrifuged. The supernatants were diluted with physiological saline to produce a series from undiluted supernatant, by doubling dilutions, to 1 : 32. Inoculations of 0.1 ml. quantities of each dilution were made into the skin of each of three guinea pigs.

No erythema was present 24 hr. after the inoculations at any of the sites where Standard vaccine had been injected.

The highest concentrations of TAG vaccine supernatant produced erythematous reactions. These decreased in diameter from 0.8 cm. at the site where the undiluted supernatant had been inoculated to 0.2 cm. where the 1 : 8 dilution had been injected. Dilution of supernatant beyond 1 : 8 and the control inoculations with TAG medium alone produced no erythema.

Discussion

Immunization of mice with TAG and Standard vaccines, followed by challenging the immunity with varying numbers of *V. comma*, has been demonstrated. The order of immunity was high. The mean LD_{50} for bivalent TAG vaccine was 32.7 ± 6.0 million *V. comma*. For bivalent Standard vaccine, the mean LD_{50} was 40.0 ± 4.7 million *V. comma*. These means were, respectively, some 300,000 and 350,000 times greater than the LD_{50} for normal mice.

The significance of the variation between the mean LD_{50}'s of the mice immunized by TAG and Standard vaccines may be assessed by determining the standard error of the differences between these means:

$$\sigma D = \sqrt{\frac{(6.0)^2}{6} + \frac{(4.7)^2}{4}} = 3.39 \text{ million } V. \textit{ comma}.$$

Thus, a difference as large as 10.17 million *V. comma* (three standard errors of the difference between the two means) might arise due to chance. Since the actual difference between the means (7.3 million *V. comma*) is smaller than the chance difference, the difference between the mean values is not statistically significant.

It may, then, be assumed that TAG vaccine protects mice as effectively as Standard vaccine.

The relative instability of TAG vaccine must be viewed as a disadvantage. Cholera vaccines are characterized by the tendency of the vibrios to autolyze during storage. TAG vaccine appeared to autolyze more quickly than Standard vaccine.

Several workers have shown that filtrates (4) and supernatants (2, 5) of *V. comma* cultures and lyzed *V. comma* (8) remain antigenic, and Fairbrother (2) has concluded that whole organisms produce better immunity than culture supernatants.

The particular disadvantage of TAG vaccine does not lie in lysis that occurs during the growth of the vibrios in the TAG medium since TAG vaccine is standardized before storage to have 8000 million *V. comma* per ml. At this time, the vaccine contains as many vibrios as Standard vaccine with an additional quantity of soluble antigens representing the vibrios that lyzed during the growth period.

It could, then, have been expected that TAG and Standard vaccines would produce equivalent levels of immunity, provided that the method of TAG vaccine production described by Ranta and McLeod (7) did not interfere with the antigenicity of *V. comma*, as the work of Haffkine (3) had implied in a report indicating a diminution in virulence of *V. comma* when cultures were aerated. However, in order to preserve whole vibrios in TAG vaccine as long as possible, some technique should be employed to limit the lysis of the vibrios during storage. The judicious use of formalin, with subsequent dilution to levels of tissue tolerance, might serve the purpose. This aspect was not explored, nor were inoculations into humans carried out with the exception of a volunteer who received three doses (0.1 ml., 0.5 ml., and 1.0 ml.) of a freshly prepared TAG vaccine subcutaneously at two-week intervals without untoward local or general reactions.

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PARALLEL STUDIES OF COMPLEMENT AND BLOOD COAGULATION

IX. THE EFFECT OF METHIONINE, CHOLINE, AND CYSTINE ON THE CHANGES INDUCED BY ETHIONINE[1]

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Abstract

Fatty livers were produced in guinea pigs by the repeated feeding or injection of ethionine; the acinar cells of the pancreas were also affected in some of the animals. Marked changes in plasma coagulability always occurred as well as a definite reduction in complement titer in which two or more of the major complement components were involved. Methionine displayed some protective effect against the fatty liver induced by the ethionine and was partially effective in controlling the coagulative changes and the decline in complement titer. In some animals, choline likewise ameliorated these conditions, whereas cystine tended to aggravate them. A combination of cystine and choline was more effective than choline alone.

Introduction

One of our major objects in undertaking this general investigation was to develop a method of producing a controlled fall in complement titer, which method could then be applied in studies of the importance of complement in determining natural resistance to disease and in various allergic phenomena. Of the drugs hitherto used, ethionine, α-amino-γ-ethylthiobutyric acid, the ethyl analogue of methionine, has seemed the most promising for this purpose since it is possible through suitable adjustment of dosage to induce a considerable fall in complement titer in guinea pigs without visibly affecting their general appearance or body weight (24). Increased dosage produces a syndrome of pathological symptoms which resembles in many respects that found in certain naturally occurring protein deficiencies (4, 7, 15). In such nutritional diseases, an increased susceptibility to various bacterial and parasitic infections is commonly observed (5). It would be of interest to know whether this low resistance bears any relationship to depressed serum complement titers.

Because of its chemical constitution, the most obvious explanation of the injurious effect of ethionine has been that it interferes directly in methionine metabolism. To determine how ethionine induces its observed effects upon the coagulative and complement systems, different aspects of methionine metabolism have been considered and an attempt made to compensate for the changes observed by supplying either methionine itself or substances known to be methionine precursors or derived from methionine *in vivo*, such as choline and cystine respectively (1, 2).

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Literature Review

Following the demonstration of Dyer (8) that ethionine fails to support the growth of rats on a methionine-deficient diet, it was likewise shown to produce a weight loss in animals on an adequate diet (29). Other common pathological findings were fatty livers, particularly in female animals (10, 11, 13, 16, 19), a chronic type of hepatic fibrosis without metamorphosis (18), marked destruction of the exocrine tissue of the pancreas (12, 14), necrosis of the kidney (32), and gross changes in the adrenals with cortical and medullary hemorrhage (13). These various conditions were aggravated when the animals were starved prior to receiving ethionine. All could be prevented or greatly alleviated by the simultaneous administration of methionine (8, 13, 16, 19, 32). Choline was without beneficial influence on the ethionine-induced fatty liver in the rat (13), suggesting that blocking of the demethylation of methionine was not the only factor involved. Stekol and Weiss (29, 31), however, found choline alone, or with cystine, maintained growth of male rats or weanling mice on an adequate diet supplemented with ethionine, whereas cystine or homocysteine failed to do so. The presence of ethionine in the diet reduced by almost 20% the amount of choline synthesized from dietary methionine labelled with deuterium in the methyl group; it did not decrease creatine synthesis (27).

Investigations with labelled materials have provided other, albeit somewhat contradictory information, in regard to the reactions of ethionine in the animal body. For example, Simpson *et al.* (28) showed that ethionine inhibited the uptake of labelled methionine *in vivo* and the conversion of methionine-sulphur to cysteine-sulphur. Twice as much methionine was required to prevent the latter inhibition as the former, and about eight times as much as was needed to protect against the appearance of fatty livers (13). Various other amino acids were not effective. Although the behavior of ethionine had been attributed at least in part to its relative resistance to de-ethylation (26), Stekol and Weiss using labelled ethionine obtained evidence suggesting that its ethyl group could be incorporated into the molecule of choline and creatine in both rats and mice (30). These workers also showed that S^{35}-labelled ethionine led to the formation of cysteine, a process which presumably involved de-ethylation of the ethionine.

In brief, therefore, the deleterious action of ethionine on tissue and blood proteins has been visualized to be of two general kinds. In the first place, ethionine may be incorporated *in toto*, in place of methionine or glycine, into the proteins themselves (20, 28), the resultant abnormal proteins having different properties from those of their methionine- or glycine-containing counterparts. In the second place, by virtue of its ethyl group, ethionine may compete with the methyl group of methionine or its precursors to form, irreversibly, products that are metabolically inactive, or possibly even toxic because of a blockade of certain essential enzymatic reactions.

Methods

Four experiments were set up, three with female guinea pigs, one with male animals. The various treated groups received ethionine alone or with methionine, choline, or cystine. Blood was collected during and after treatment and the diluted plasma tested for coagulability, the serum for complement content. Microscopic examinations were made of specimens of liver, kidney, pancreas, and adrenals of animals that died as a result of cardiac bleeding or that were sacrificed at the end of the experiment.

Treatment of Guinea Pigs

Experiment 1

Fifty female guinea pigs, weighing 728 to 844 gm., were divided on the basis of weight into five groups of 10 animals each. The first group was left untreated, the other four groups were given daily, subcutaneous injections of 100 mgm. ethionine for a 15-day period. In addition to ethionine, the third group received daily injections of 100 mgm. *dl*-methionine, the fourth group the same amount of choline chloride, the fifth of *l*-cystine. The animals were bled after 10 days' feeding, and again five days later, no further injections having been given in the intervening period. Most of the animals injected with ethionine, with or without supplement, showed some loss of weight (mean loss 14 gm.) but much less than had been observed after the repeated injection of carbon tetrachloride (23).

Experiment 2

Thirty female guinea pigs were divided into three groups of 10 animals of approximately comparable weight. The first group received 100 mgm. ethionine only; the second ethionine (100 mgm.) and choline chloride (100 mgm.); the third ethionine (100 mgm.), choline chloride (100 mgm.), and *l*-cystine (50 mgm.). Five additional animals were injected with 50 mgm. *l*-cystine without ethionine. With the exception of ethionine, all supplements were injected daily; after six daily doses of ethionine, it was necessary because of the poor condition of the animals to give the drug on alternate days only. All guinea pigs were bled after eight doses of ethionine and 10 of the other supplements.

Experiment 3

Fifty male guinea pigs, weighing approximately 600 to 900 gm., were divided by weight into five groups of 10 each. The first group was left untreated, the other four groups received a total of 13 subcutaneous injections of 50 mgm. ethionine, the first nine injections daily, the remaining four at two-day intervals. In addition to the ethionine, the third group was given daily doses of 100 mgm. methionine, the fourth of 50 mgm. choline chloride, and the fifth of 50 mgm. glycine. Thus during the latter part of the experiment when the ethionine was being injected at two-day intervals, the various supplements were being given daily. Blood was collected following the ninth and the last doses; the untreated controls were bled at the same times.

Experiment 4

This experiment was designed to ascertain whether the administration of glucose would assist in the maintenance of complement titers during ethionine injection. Thirty female guinea pigs, 580 to 880 gm. in weight, were divided into three groups of 10 each. The first group was left untreated, the second and third groups were injected with 100 mgm. ethionine daily for nine days, then on alternate days until a total of 16 doses had been given. The third group was fed a solution containing approximately 1.6 gm. glucose daily for the entire period. Blood was taken after 10 and 16 days' treatment.

During the first 10 days, the untreated control group showed a mean gain in weight of 19 gm., the ethionine controls a mean loss of 32 gm., the ethionine and glucose treated group a mean loss of 19 gm. In the period when ethionine was being administered every second day and glucose daily, the weight of the treated animals remained stationary or increased slightly.

Coagulation Tests

Two-stage prothrombin determinations on plasma diluted 1:25 were made using the same technique as has been employed in our earlier investigations (24). The clotting time was recorded under three different sets of conditions; stage I, without added fibrinogen; stage II with added fibrinogen; and stage III (modified) with added fibrinogen and diluted (1:50 or 1:100) prothrombin-free guinea-pig serum (33). Comparison of the clotting times recorded in the three tests therefore indicated the relative accelerating effect of adding fibrinogen and/or prothrombin-free guinea pig serum (P.F.S.) to the diluted plasma. It thus provided a rough estimate of the relative deficiency of the particular test plasma in fibrinogen, prothrombin, and Ac-globulin (labile factor) as compared with the diluted plasma of the normal guinea pigs.

Complement-fixation Tests

All sera were titrated in serial amounts to determine the amount required for 50% hemolysis; the whole complement or C′ titers were calculated in units per ml. from these values. The titers of the four major complement components, C′1, C′2, C′3, and C′4, were determined by testing serial dilutions with varying amounts of suitable guinea-pig test reagents lacking the respective component. In the sera of our untreated animals, C′2 has ordinarily been found to be the complement component present in lowest titer. C′3 titers have tended to run higher, particularly in the spring and summer.

Protein Analyses

Determinations of total serum protein content were made by a nesslerization method, and the albumin and globulins separated by fractional precipitation with 27.2 and 19.9% sodium sulphate (17).

Results

Histopathology

In these experiments, the major changes observed were in the liver; pancreatic damage involving the acinar cells was noted in a smaller number. In a few animals, hemorrhagic kidneys and enlarged adrenals were found on gross examination.

Experiment 1

Marked fatty changes were observed in the livers of all ethionine-treated guinea pigs examined. Practically all of the liver cells appeared swollen and contained numerous fine vacuoles; some cells showed a single very large vacuole. The livers of the guinea pigs given choline as well as ethionine, or both *dl*-methionine and ethionine, were somewhat less affected. In two of six of the former group of animals, the livers appeared almost normal, four showed considerable fatty change but the vacuoles tended to be smaller and the degree of change less marked than in the ethionine-treated controls. Two animals from the group given ethionine and methionine were examined, the liver of one seemed essentially normal; moderate numbers of vacuoles were seen in the cells at the periphery of the lower lobules of the other. About one fifth to one third of the cells in the livers of seven guinea pigs given both *l*-cystine and ethionine were in the "signet ring" form.

Experiment 2

Although the daily injection of choline chloride in twice the amount employed in the previous experiment did not prevent the development of considerable fatty degeneration of the liver, the proportion of the animals surviving was greater than in the ethionine controls. Five of the 10 animals injected with both choline and cystine in addition to ethionine, were examined; three displayed pancreatic damage. In one, the liver appeared normal; a diffuse fatty degeneration was noted in a second; cloudy swelling and rarefaction of the liver cytoplasm in the other three. The pancreas of six or seven animals given ethionine alone appeared damaged, the acinar cells swollen and in many instances vacuolated.

Experiment 3

The livers of male guinea pigs after daily injection of 50 mgm. ethionine showed a somewhat less severe fatty condition than females that had received a similar dosage. Following 13 injections however, at least two-thirds of the liver cells had usually been reduced to the "signet ring" form with many of the cells in the remaining third occupied by small vacuoles. Neither choline nor glycine protected appreciably against this accumulation of fat.

Experiment 4

The daily feeding of 1.6 gm. glucose to guinea pigs was likewise ineffective in preventing the development of fatty changes in livers of female guinea pigs during daily injection of 50 mgm. ethionine; the liver cells showed slight to

severe fatty degeneration. In one of the ethionine controls, a marked destruction of the acinar cells of the pancreas was observed. One of this control group and two of the glucose-fed animals exhibited pathological changes in the kidney as well as in the liver.

Changes in Plasma Coagulability

Experiment 1

All guinea pigs after 10 injections of ethionine exhibited a marked increase in plasma clotting time, Table I. In stage I, this ranged from 387 sec. to over an hour, as compared with a range of 121 to 187 sec. for the untreated controls. The addition of fibrinogen in stage II reduced the clotting times of the individual plasmas by 304 sec. to more than 50 min. The mean value of 182 sec., although significantly shorter ($P > 0.001 < 0.01$) than the stage-I clotting times of the same specimens, was still significantly longer than the mean value for the control group: 41 sec., range 26 to 48 sec. A further reduction of

TABLE I

THE RELATIVE EFFICACY OF METHIONINE, CHOLINE, AND CYSTINE SUPPLEMENTS IN PREVENTING THE CHANGES IN THE CLOTTING TIME OF DILUTED PLASMA INDUCED BY THE INJECTION OF ETHIONINE

Expt.	Group	Date of bleeding	Number of plasmas tested	Clotting time (sec.)					
				Stage I		Stage II*		Stage II (modified)*	
				Mean	σ	Mean	σ	Mean	σ
		1951							
1	Untreated controls	13/8	10	164	20	51	8	50	7
	Ethionine controls	"	8	1448**	1163	182	123	139	74
	Methionine–ethionine	"	10	389	139	102	31	82	20
	Choline–ethionine	"	6	656	413	86	52	76	46
	Cystine–ethionine	"	3	635	437	267	154	152	63
1	Untreated controls	18/8	3	169	26	51	3		
	Ethionine controls	"	3	264	17	62	5		
	Methionine–ethionine	"	4	251	43	77	15		
	Choline–ethionine	"	4	234	65	80	14		
	Cystine–ethionine	"	3	234	45	63	10		
2	Untreated controls	14/9	6	209	28	32	9	30	8
	Ethionine controls	"	5	512	282	90	41	60	26
	Choline–ethionine	"	4	450	175	69	31	53	30
	Choline–cystine–ethionine	"	5	284	194	149	91	64	18
	Cystine controls	"	5	220	29	59	16	29	7

* *The total clotting time is the value given plus the 45 sec. arbitrarily allowed for conversion of prothrombin to thrombin.*

** *Clots tended to be stringy and atypical in appearance.*

10 to 104 sec. was effected in the clotting times of plasma from individual ethionine-treated animals in the presence of added P.F.S. as well as fibrinogen: the difference in the mean clotting times in stage II and stage II (modified) was not statistically significant, however. The addition of P.F.S. had no effect on the clotting time of normal plasma in stage II (modified).

Of the supplements, none was effective in maintaining plasma coagulability at normal levels when ethionine was being injected daily. Although the mean stage-I clotting times for the methionine, choline, and cystine supplemented groups were shorter by 1059, 792, and 813 sec. respectively than the mean value for the ethionine control, only in the case of the methionine–ethionine group was this difference of probable statistical significance ($P > 0.01 < 0.02$). The addition of fibrinogen in stage II reduced the mean clotting time of the plasma of the choline–ethionine group of guinea pigs by 570 sec. as compared with a reduction of 287 sec. in the methionine–ethionine group and 1266 sec. in the ethionine controls. The mean stage-II clotting times of the methionine–ethionine and ethionine control groups were still significantly longer than that of the normal group ($P < 0.001$); the elevation of 35 sec. in the mean value for the choline-ethionine group was not of statistical significance ($P > 0.05$). In other words, when the deficiency in fibrinogen had been compensated for, plasmas of the choline–ethionine treated animals were shown to have a more nearly normal prothrombin content than the plasmas of guinea pigs of the methionine–ethionine group. The addition of both P.F.S. and fibrinogen in stage II (modified) brought the clotting times of both of these groups to almost normal levels, whereas the clotting times of the ethionine controls were still significantly elevated ($P > 0.001 < 0.01$).

These experiments indicated therefore that ethionine injection produces a significant fall in the fibrinogen and prothrombin content of guinea-pig serum, and in certain individuals a concomitant decline in Ac-globulin (labile factor). The methionine supplement was partially effective in controlling the decline in fibrinogen content; both methionine and choline aided in maintaining prothrombin closer to the normal level. The cystine supplement had no apparent protective effect either on fibrinogen or on prothrombin and may even have had a deleterious influence upon Ac-globulin production or functioning.

The plasma from blood drawn five days after the last injection of ethionine had stage-I clotting times shorter than those of the previous bleeding from the same animals, but the mean times were still significantly longer than normal ($P > 0.001 < 0.01$). None of the previously given supplements resulted in more rapid recovery in plasma coagulability. The elevation of the mean stage-II clotting time of the ethionine controls over that of the untreated controls was of lesser magnitude, suggesting that recovery in prothrombin was taking place more rapidly than in fibrinogen.

Experiment 2

The mortality in this series of guinea pigs was higher than in the preceding one, necessitating a reduction in the number of injections of ethionine. Plasma coagulability was found to be less affected than in the previous experiment as

a result of this lower total dosage. Nevertheless, the mean stage-I clotting time of the ethionine controls was 303 sec. longer than that of the untreated controls, a difference of probable statistical significance ($P > 0.02 < 0.05$). The mean stage-I clotting time of the choline–ethionine group was also significantly depressed, but that of the choline–cystine–ethionine group was not significantly longer than normal, suggesting that the combination of choline chloride and cystine had been more effective as supplements than the former alone. The stage-II and stage-II (modified) clotting times of the ethionine controls were 58 and 30 sec. longer respectively than those of the normal group, differences of probable statistical significance. The mean stage-II clotting time of choline–cystine–ethionine group was 117 sec. longer than the normal mean value, but not significantly longer than that of the ethionine controls or the choline–ethionine group ($P > 0.05$). The addition of P.F.S. produced a further reduction of 85 sec. in the mean clotting time of this treated group as compared with 2 sec. in the normal controls, 30 sec. in the ethionine controls, and 16 sec. in the choline–ethionine group.

That cystine injection might have had a depressive effect on Ac-globulin production or activity was also suggested by the results observed in the cystine controls. Whereas the stage-I and stage-II (modified) clotting times of these five plasmas were comparable to those of the six normal plasmas tested in the same day, their mean stage-II clotting times were significantly longer ($P > .001 < .01$). That is, P.F.S. as well as fibrinogen was needed to bring the clotting times in the cystine controls to the normal level.

Changes in Complement Titers

Experiment 1

The repeated injection of ethionine induced a marked fall in whole complement titers in all guinea pigs so treated (Table II); the mean C′ titer was less than one fourth of that of the untreated controls. All four of the major components of complement were reduced but not to the same degree either in relation to each other in the same animal or in individual animals. The most marked reduction was in C′2 and C′3, although C′1 and C′4 fell to very low levels in some animals.

When the guinea pigs were injected with methionine or choline chloride as well as ethionine, a lesser drop in mean C′ titers occurred; cystine had no protective effect. The mean C′ titer of the methionine–ethionine group was significantly higher than that of the ethionine controls ($P \leqq 0.01$), The difference between the C′ titer of the choline–ethionine group and the ethionine controls, 203 units per ml., was of probable statistical significance ($P \leqq 0.05$).

Methionine injection maintained C′1 titers at normal levels in ethionine-treated animals. The mean C′1 titer in the choline–ethionine group, although higher than those of the ethionine controls was not significantly so. The low C′1 titers observed in the cystine–ethionine group were comparable to those recorded for many of the ethionine control animals. In relation to C′2, choline had somewhat greater protective properties than methionine; the

TABLE II

THE EFFECT OF METHIONINE, CHOLINE, AND CYSTINE ON THE DECLINE IN COMPLEMENT TITERS INDUCED BY ETHIONINE (EXPT. 1)

Group	Complement component	Titer (units/ml.)					
		Bl. 13/8/51			Bl. 18/8/51		
		No. tested	Mean	σ	No. tested	Mean	σ
Untreated controls	C′	10	1942	266	3	1650	127
Ethionine controls		8	435	146	3	958	312
Methionine–ethionine		10	836	402	4	976	269
Choline–ethionine		6	668	227	4	1375	230
Cystine–ethionine		5	355	172	3	913	67
Untreated controls	C′1	10	2782	743	3	2167	575
Ethionine controls		8	1085	764	3	3017	272
Methionine–ethionine		10	2526	976	4	2878	439
Choline–ethionine		6	1740	433	4	3122	415
Cystine–ethionine		5	522	136	3	3333	0
Untreated controls	C′2	10	2290	1161	3	1672	245
Ethionine controls		8	571	218	3	1144	248
Methionine–ethionine		10	1047	569	4	1398	331
Choline–ethionine		6	2041	714	4	2080	455
Cystine–ethionine		5	571	133	3	1170	424
Untreated controls	C′3	10	2145	379	3	2495	770
Ethionine controls		8	733	404	3	1235	344
Methionine–ethionine		10	1736	353	4	1497	244
Choline–ethionine		6	1334	429	4	1874	418
Cystine–ethionine		5	497	286	3	3333	0
Untreated controls	C′4	10	>10,000		3	>10,000	
Ethionine controls		8	2839	2240	3	>10,000	
Methionine–ethionine		10	>9300		4	>10,000	
Choline–ethionine		6	2423	1200	4	>10,000	
Cystine–ethionine		5	1450	380	3	>10,000	

mean C′2 titer for this choline–ethionine group fell within the normal range, and was significantly higher than of the methionine–ethionine group ($P > 0.01 < 0.02$). The mean C′2 titer of the methionine–ethionine group was nevertheless 476 units per ml. higher than that of the ethionine controls, a difference of probable significance ($P > 0.02 < 0.05$).

Both choline and methionine protected against the fall in C′3 titer that accompanied ethionine injection; the mean C′3 titers of both of these groups were significantly higher than that of the ethionine controls ($P > 0.001$), even though both were significantly lower than the mean C′3 titer of the

normal controls. In the cystine–ethionine group, the C′1, C′2, and C′3 titers were comparable to or lower than those of the ethionine-control animals. C′4 titers fell sharply in all ethionine-treated animals except those receiving the methionine supplement. The most marked decline was observed in the cystine–ethionine group.

After a rest period of six days, without further injection of ethionine or of the various supplements, an improvement in C′ titers was recorded, particularly in the group that had previously received choline as well as ethionine. By this time C′1 and C′4 titers had returned to normal levels in all animals; the recovery in C′2 and C′3 titers was somewhat slower.

Experiment 2

In this experiment in which the total dosage of ethionine was reduced, the decline in complement titers was not so striking (Table III). The choline supplement alone was not effective in preventing this decline in C′, but a combination of choline and cystine seemed to afford more protection; the difference in mean C′ titers in the choline–cystine–ethionine and ethionine control groups was of probable significance ($P > 0.01 < 0.02$). Cystine alone had no apparent influence on C′ titers.

The fall in C′1 titers during ethionine treatment, though relatively less than in the previous experiment was of probable significance ($P > 0.02 < 0.05$). Choline alone did not prevent this decline, but in animals given both choline and cystine as well as ethionine, the C′ titers remained at normal levels. The combination of the two supplements as shown earlier also prevented coagulative changes more effectively than either alone. A definite fall in C′2 and C′3 titers was recorded in the ethionine control and choline–ethionine groups. The C′2 titers of the choline–cystine–ethionine group remained within the normal range. The mean C′3 titers of this group were significantly below normal ($P > 0.001 < 0.01$) but 557 units per ml. higher than those of the ethionine controls, a difference of probable significance ($P \leqq 0.05$), and 904 units per ml. higher than the C′3 titer of the choline–ethionine-treated guinea pigs, a highly significant difference ($P < 0.01$).

Experiment 3

Although the liver changes produced by ethionine in male guinea pigs are in general less extensive than those observed in females, the drug depresses complement titers markedly in the former as well as the latter sex (24). Judged on the basis of complement titers of specimens taken on 10/3, after nine daily doses of ethionine, none of the supplements, methionine, choline, or glycine had been effective in preventing a fall in the titer of whole complement or of the four major components. Subsequently when ethionine was given on alternate days and the supplements daily, an increase occurred in the complement activity of the serum of all treated animals. Mean C′ titers of the 19/3 bleedings were still significantly below that of the normal group in all except the methionine–ethionine group. The mean C′ titers of the

TABLE III

THE EFFECT OF VARIOUS SUPPLEMENTS UPON THE DEPRESSION IN COMPLEMENT TITER PRODUCED BY THE CONCOMITANT INJECTION OF ETHIONINE

Experiment	Group	Date of bleeding	Number of sera tested	Titer (units/ml.)							
				C′		C′1		C′2		C′3	
				Mean	σ	Mean	σ	Mean	σ	Mean	σ
		1951									
2	Untreated controls	14/9	6	1420	253	2976	504	1588	276	2320	438
	Ethionine controls	"	5	531	175	1736	1091	915	507	915	561
	Choline–ethionine	"	4	418	100	1435	429	792	347	568	244
	Choline–cystine–ethionine	"	5	989	285	3388	448	1502	314	1472	87
	Cystine controls	"	5	1540	212	2212	434	1966	288	1893	394
		1952									
3	Untreated controls	10/3	10	1462	233	2241	545	1912	353	1481	398
	Ethionine controls	"	10	636	155	1328	430	712	208	685	261
	Methionine–ethionine	"	10	646	170	1150	323	772	225	571	263
	Choline–ethionine	"	9	679	144	1316	431	742	172	648	116
	Glycine–ethionine	"	8	677	147	1342	243	798	253	730	160
3	Untreated controls	19/3	10	1531	296	2604	618	2172	402	1583	260
	Ethionine controls	"	8	971	322	1812	658	1251	505	1116	466
	Methionine–ethionine	"	10	1436	140	1612	679	1492	324	1520	367
	Choline–ethionine	"	6	974	191	1217	371	1470	309	1138	305
	Glycine–ethionine	"	7	789	253	1893	793	1482	462	1158	542
4	Untreated controls	28/4	10	1599	224	2352	254	1405	434	1629	371
	Ethionine controls	"	9	478	233	861	363	641	132	692	597
	Glucose–ethionine	"	7	477	169	1381	750	592	347	836	273

choline–ethionine and glycine–ethionine groups were comparable to that of the ethionine control. None of the supplements gave definite evidence of accelerating the return of the C′1 or C′2 titers to normal levels; C′3 titers fell within the normal range in the methionine–ethionine group.

Experiment 4

In view of the findings of Farber *et al.* (12, 13) that glucose feeding prevented the development of a fatty liver condition in ethionine-injected rats, it seemed worthwhile to determine whether it acted similarly in guinea pigs, and if so whether this would be reflected in a lesser decline in complement titers. As administered, however, its therapeutic effect on the liver was less definite than that recorded in rats, and as shown in Table III, complement titers in the glucose–ethionine group were comparable to those of the ethionine controls.

Changes in Serum Protein Values

Serum protein analysis were made on the first series of blood specimens from 15 male guinea pigs in the third experiment. These showed that ethionine injection had resulted in some reduction in total serum protein content. The mean total value for the four ethionine controls, 5.87 gm./100 ml. ($\sigma = 0.17$), was somewhat lower than that of the four untreated controls, 6.69 gm./100 ml. ($\sigma = 0.27$), a difference of statistical significance ($P > 0.001 < 0.01$). The total protein values of sera from the methionine–ethionine, choline–ethionine, and glycine–ethionine groups were comparable with those of the untreated, Table IV, suggesting that these supplements may have been of assistance in this respect even though they had not seemed so in regard to complement activities (Table III).

As has been observed in our earlier studies, the serum albumin values of the ethionine-treated guinea pigs remained at normal levels; the mean value for the untreated controls was 3.90 gm./100 ml. ($\sigma = 0.27$), for the ethionine controls 3.87 gm./100 ml. ($\sigma = 0.22$). The mean total globulin value for the ethionine controls, 1.99 gm./100 ml. ($\sigma = 0.14$), was lower than the mean normal value of 2.79 gm./100 ml. ($\sigma = 0.04$), a highly significant difference ($P = <0.001$). The decrease in total serum protein accompanying ethionine injection represented therefore a fall in globulin rather than in albumin. Of the two globulin fractions, euglobulin was reduced more consistently than pseudo-globulin; the mean serum euglobulin value for the untreated controls was 0.55 gm./100 ml. ($\sigma = 0.22$), for the ethionine controls 0.12 gm./100 ml. ($\sigma = 0.09$).

As a result of the tendency for total globulin to fall and albumin to remain constant, the albumin–globulin quotient ranged relatively higher for sera from the ethionine-treated guinea pigs; mean 1.95 ($\sigma = 0.22$), as compared with a mean normal value of 1.38 ($\sigma = 0.29$). The difference between these two means was of probable statistical significance. The A/G quotient, however, never ranged as high in these male animals as in sera from a number of the

female guinea pigs previously analyzed (24). The one animal from the choline–ethionine group and two of the three methionine–ethionine animals examined had A/G ratios comparable with those of the normal animals and lower than any recorded for the ethionine controls. Two of three guinea pigs from the glycine–ethionine group, on the other hand, had A/G ratios closer to those of the ethionine controls. The nonprotein nitrogen values for the various groups of sera were relatively comparable.

The complement titers which have also been included in Table IV, show that no direct relationship existed between these titers and the total protein or total globulin content of the respective sera, except that when the latter values were depressed the former were also low; the converse was not consistently observed. Some sera, those of guinea pigs Nos. 727, 645, 719, and 745 for example, had relatively depressed complement titers and a total protein content within the normal range. In three of these four sera the proportion of albumin: globulin was increased.

Comparison with Carbon Tetrachloride Treated Animals.

Although both carbon tetrachloride (23,24) and ethionine produced a marked decline in the coagulative and complement activities of the blood of treated guinea pigs, their effects on the individual components of the two systems differed. Whereas both reagents reduced fibrinogen, Ac-globulin, and second component of complement, prothrombin fell more markedly after carbon tetrachloride treatment, and C′1 and C′3 following ethionine administration.

TABLE IV

SERUM PROTEIN VALUES OF UNTREATED AND TREATED MALE GUINEA PIGS

Tag No. of G.P.	Group	C′ titer, units/ml.	Non-protein nitrogen, mgm./100 ml.	Total protein, gm./100 ml.	Albumin, gm./100 ml.	Pseudo-globulin, gm./100 ml.	Eu-globulin, gm./100 ml.	A/G ratio
366	Untreated controls	1720	35.5	7.02	4.25	2.46	0.31	1.53
N-1		1670	35.5	6.70	3.85	2.33	0.52	1.35
731		1320	32.5	6.67	3.89	2.18	0.60	1.33
717		1280	30.0	6.36	3.60	1.98	0.79	1.30
739	Ethionine controls	590	28.0	5.91	3.70	2.00	0.21	1.68
736		250	33.0	5.79	3.86	1.81	0.12	2.00
723		543	32.5	6.09	4.19	1.90	—	2.21
713		833	40.0	5.68	3.74	1.78	0.16	1.92
727	Methionine–ethionine	920	33.0	6.73	4.46	2.31	—	1.93
645		850	40.5	7.15	4.22	2.68	0.25	1.44
691		567	43.0	6.13	3.40	2.73	—	1.25
742	Choline–ethionine	893	30.0	6.37	3.40	2.18	0.79	1.14
719	Glycine–ethionine	455	35.0	6.34	4.17	1.91	0.26	1.93
744		833	40.0	6.15	3.74	1.78	0.63	1.55
745		769	30.0	6.68	4.60	1.51	0.57	2.21

Considerable variability in C′4 values was noted in all treated groups. In general this component was not appreciably affected until a marked decrease in C′2 titer was demonstrable. Serum proteins were not affected in the same manner by the two agents. After carbon tetrachloride there tended to be a fall in total serum protein and albumin and an increase in euglobulin. Ethionine in moderate dosage induced some decrease in total serum protein but little change in albumin; globulin decreased significantly, the euglobulin fraction often falling to very low levels. This variation in serum-protein pattern was reflected in the relative changes in C′1 titers induced by the two agents. C′1, a euglobulin precipitated by 1.39 *M* ammonium sulphate (21), tended to decline after ethionine injection but seldom following carbon tetrachloride treatment. This apparent correlation may have been only an interesting coincidence since prothrombin (25) with similar solubilities to C′1 (22) was more perceptibly reduced by carbon tetrachloride than by ethionine injection. The relative effects of choline and methionine also differed in guinea pigs treated with carbon tetrachloride and ethionine.

Discussion

The foregoing studies have indicated that methionine will protect to some degree against the changes in the coagulability of plasma and the fall in complement titer produced in guinea pigs by the administration of its ethyl analogue, ethionine. The fact that methionine did not completely prevent these effects may have been due to irreversibility of certain of the intermediate ethylated products formed or its failure to replace ethionine in abnormal proteins into which the latter had been incorporated. The beneficial effect of choline was probably related primarily to its lipotropic action on the liver, and secondarily to its role as a donor of methyl groups for the synthesis of methionine or its precursors. The augmented effect when cystine as well as choline was added suggested that a deficiency of this amino acid may also have been created.

In view of the relatively extensive quantitative and qualitative changes in the protein components of the coagulative and complement systems, and the marked degree of fatty degeneration of the liver observed, a concomitant drop in the total protein content of the serum was expected. In the present series, and in the earlier one which had included sera from both male and female guinea pigs, some, but not all of the animals showed a decrease in total serum protein. This decrease involved a fall in globulin rather than in albumin, such as is observed following the administration of carbon tetrachloride or chloroform, or in certain forms of liver disease. If abnormal, ethionine-containing serum proteins were being formed, they may have been precipitated in the albumin fraction, that is with proteins relatively low in cystine and glycine, rather than with the globulins which are relatively high in cystine, glycine, and hydroxy amino acids (3, 6). First component of complement, decreased as result of ethionine treatment, has been shown to have an amino acid composition similar to that of other globulins previously analyzed (9).

Fibrinogen, likewise affected by ethionine administration, has a high glycine and methionine content and a low cystine value as compared with albumin (3). It might be visualized that such disturbances in methionine metabolism might extend to the synthesis of other important globulins, notably enzymes, and of certain hormones.

Acknowledgments

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AUTOLYSIS IN NORMAL AND PATHOLOGICAL CEREBROSPINAL FLUIDS[1]

By Ernest Kovacs

Abstract

Variations in acid soluble inorganic phosphorus concentration of some 850 specimens of cerebrospinal fluid (CSF) were assayed before and after incubation, under sterile and standardized conditions. These phosphorus changes are the consequences of interaction of enzymes and phosphorus-containing organic material present in the CSF. Characteristic patterns were noted in the different etiological groups. Normal CSF exhibited no phosphorus changes after incubation, nor did specimens from neurologically normal persons; exceptions were rare. On the other hand in the CSF of acute bacterial meningitides a great increase of phosphorus was usually evident. No changes were observed in a large number of poliomyelitis specimens. This similarity to normal may be due to quite dissimilar causes. In the syphilis group, a great increase of phosphorus was found in active neurosyphilis. In convulsive disorders, idiopathic epilepsy manifested the highest increase. CSF's of post-traumatic syndrome patients showed uniformly high phosphate release. Brain tumor specimens manifested moderate changes.

Introduction

The subject of autolysis in general has been extensively reviewed by Levene (18) and Haehn (6). Kafka (10) was the first to make a systematic study of autolysis in the CSF. He tried two different approaches: assays of "endoproteases" and the investigation of antienzymes which inhibit autolysis. He was not able, however, to demonstrate autolysis in the incubated CSF's. It was concluded that the slight increase of nitrogen-containing material was not due to autolysis. Wiechman (26) on the other hand maintained that the increase of amino acids in the CSF was the consequence of autolysis. We wish to report upon behavior of acid soluble inorganic phosphorus in incubated buffered CSF, under sterile and well controlled conditions. The simple and exact determination of the changes in this important constituent may serve as a clue to the question of the nature of autolysis in the CSF. Our findings are reported in a large number of normal, neurologically normal, and pathologic specimens.

Methods

Experimental Technique

The quantity of CSF used routinely was 0.2 ml. Occasionally smaller and larger volumes were assayed, but 0.2 ml. was found the most convenient. This volume was pipetted into a glass-stoppered sterile bottle, along with 10 ml. of veronal–acetate buffer, Michaelis (21), pH $\pm$ 7.2 to 7.4. Two drops of chloroform or toluene were added as preservative and the system incubated

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for 24 hr. at 37° C. As controls, the following were employed: CSF alone and heat (56° C.) inactivated CSF in buffer in the quantities used in the preparation of the incubated system.

Determination of Zero Values and End Values

Zero value or starting value is the value of the phosphorus concentration before incubation. End value means similarly the value after incubation. In order to assure a reliable zero value the CSF and buffer are mixed in an ice bath and aliquots of this mixture, which correspond to the incubated system, are pipetted to equal volumes of 10% chilled trichloroacetic acid, mixed, allowed to stand for 10 min. in the ice bath with occasional vigorous shaking, and filtered through Whatman's No. 40 or No. 42 acid treated filter paper. Aliquots of the filtrate were used for duplicate determination of the acid soluble inorganic phosphorus by the method of Fiske and Subbarow (5) as modified by King (14). In a large number of specimens the acid soluble total phosphorus was also determined after perchloric acid digestion, as described by King (14). In the measurement of the end concentrations after incubation, the same procedures were carried out, namely initial chilling, precipitation, filtration, and determination. Color readings were made routinely with a Klett-Summerson photocolorimeter and occasionally with a Beckman spectrophotometer at 660 μ.

The effect of the physiological buffer of Michaelis (21), physiological saline, changes in nonbuffered CSF, Mg ++ and Ca ++ activation in 0.01% final concentration, temperature, pH, and freezing were also investigated. Of the total specimens examined approximately 25% were used in the fresh state and the remainder after freezing at −25° C. and thawing in the icebox before use. Cell free, centrifuged specimens were used with the exception of samples from neurosyphilis and the majority of poliomyelitis specimens. Strict asepsis was maintained throughout and sterility tests made to control the technique.

Results

Over 2500 determinations were made on more than 850 individual CSF samples. In order to economize space, characteristic examples only are shown and grouped in Table I. A synopsis of the total bulk of results is given in Table II and in figures.

In the 100 specimens of normal and neurologically normal cases there was no change in the acid soluble inorganic phosphorus concentration after 24 hr. incubation at 37° C. (Table I, Group A). There were, however, rare exceptions in the neurologically normal group as, for example, upper respiratory infections (two cases), cervical adenitis (one case), pyrexia of unknown origin (two cases) which showed considerable increase (Table II, Group *b*).

In the meningitis cases of various etiology (200 specimens) of Table I, Group B, a high increase in the acute stage of the disease was found. Subacute and chronic cases showed less or no change. In the five *E. coli* meningitis

TABLE I

PHOSPHORUS CHANGES IN THE CSF AFTER INCUBATION

Name	Diagnosis	Lab. notes	Phosphorus in μgm. per 2.5 ml. of system	
			0 values	End values
Group A—Normals, neurologically normal persons: 100 cases				
G. S.	Normal case	Neg. lab. findings	3.0	3.2
M. N.	" "	" " "	3.0	3.2
R. M.	Acute osteomyelitis	" " "	3.0	3.0
P. M.	Cervical adenitis	" " "	4.0	8.0
Group B—Meningitides: 200 cases				
E. L.	Meningococcal meningitis	High cell count, high proteins	2.0	4.0
S. D.	Pneumococcal meningitis	" " " " "	6.0	12.0
T. B.	Purulent meningitis	" " " " "	2.0	5.0
I. M.	Influenzal meningitis	" " " " "	5.0	12.0
B. S.	*E. coli* meningitis	" " " " "	8.0	4.0
Group C—Poliomyelitis and other virus diseases: 160 cases				
O. S.	Nonparalytic poliomyelitis	492 Ly Pa++	4.1	4.0
A. M.	Paralytic poliomyelitis	170 Ly Pa+	2.2	2.1
J. M.	Bulbar poliomyelitis	53 Ly Pa++	2.6	2.6
R. D.	Lymphocytic choriomeningitis	417 Ly Pa+	6.6	4.0
M. W.	Infectious polyneuritis	0 cells Pa+++	5.0	7.0
Group D—Syphilis and neurosyphilis: 240 cases				
L. D.	Primary syphilis	Ser. Wass. R. pos., CSF Wass. R. neg.	7.0	7.0
G. S.	Secondary syphilis	Ser. Wass. R. pos.	2.0	3.0
J. L.	Early latent syphilis	Ser. Wass. R. doubtful, CSF Wass. R. neg.	3.0	6.0
E. R.	Late latent syphilis	Ser. Wass. R. pos., CSF Wass. R. neg.	3.1	3.0
P. C.	Neurosyphilis	Ser. Wass. R. doubtful, CSF Wass. R. strongly pos.	2.8	7.0
P. H.	Paretic type	Ser. Wass. R. pos., CSF Wass. R. moderately pos.	1.5	5.0
T. M.	Tabetic type	Ser. Wass. R. pos., CSF Wass. R. strongly pos.	5.0	7.0
F. B.	Taboparetic type	Ser. Wass. R. doubtful, CSF Wass. R. negative	3.0	5.0
Group E—Convulsive disorders: 50 cases				
L. S.	Idiopathic epilepsy	Neg. CSF findings	3.0	6.0
B. L.	Idiopathic epilepsy	" " "	15.0	18.0
M. W.	Febrile convulsion	" " "	5.0	5.0
Group F—Miscellaneous neurological and psychiatric cases: 100 cases				
R. A.	Porphyriasis	Cells: 1700, Pa+	23.0	47.0
E. M.	Pituitary adenoma	Proteins 78 mgm. %	2.0	3.0
M. A.	Post-traumatic syndrome	Neg. lab. findings	4.0	8.0
S. E.	Schizophrenia (after shock therapy)	Neg. lab. findings	2.0	6.0

cases examined a decrease of phosphate values was found, except in one, in which moderate increase was noted. Bacterial meningitides are considered in the group illustrated. Centrifuged, cell free CSF's were used.

The poliomyelitis cases (140), in Table I, Group C, present an interesting picture. No changes of acid soluble inorganic phosphorus concentration was found in the overwhelming majority. This result is the most significant since noncentrifuged CSF was used in almost all of the assays of this group. In the detailed analysis of the polio group as may be seen in Table III there are 25 exceptions out of 140 specimens; that is, in 82% of the cases there was no change and in 18% there was some change after incubation. In the

TABLE II

PERCENTAGE OF CHANGES OF INORGANIC PHOSPHORUS IN 850 SPECIMENS OF CSF

Group	Number of cases	Range of inorganic phosphorus change in per cent
(*a*) Normals	30	0 – 10+
(*b*) Neurologically normals	65	10 – 25+
	5	25 – 150+
(*c*) Meningitides	20	10 – 25+
	10	25 – 50+
	165	50 – 200+
E. coli meningitides	1	100+
	4	25 – 100–
(*d*) Poliomyelitis	113	No change
	12	0 – 25+
	13	15 – 100+
	1	100+
	1	150+
Other virus diseases	6	0 – 100+
	11	0 – 100–
(*e*) Syphilis	65	0 – 100+
Neurosyphilis	14	0 – 12+
	126	30 – 250+
(*f*) Convulsive disorders:		
(1) Genuine epilepsy	2	No change
	22	0 – 100+
(2) Symptomatic convulsions	9	0 – 38+
	7	0 – 25–
(*g*) Miscellaneous neurological conditions:		
(1) Tumors	19	0 – 100+
	6	0 – 100–
(2) Psychiatric cases	4*	50 – 150+
	21	0 – 20
(3) Post-traumatic cases	15	50 – 100+
(4) Other neurological conditions	5	35 – 100+
	40	10 – 50+

\+ Increase.
– Decrease.
* Organic psychoses (three cases).

nonparalytic form exceptions occurred in 25%, in the paralytic 12%, in bulbar cases 20%. Polioencephalitis showed no exceptions; there were, however, only four cases. The four lymphocytic choriomeningitis cases showed slight increase or decrease, but the two Guillain-Barré specimens and the four mumps encephalitis cases showed definite increase.

The samples of the large syphilis group of 240 cases (Table I, Group D) showed marked differences in the change of phosphorus values at different stages of the disease. In the CSF of primary syphilis there was in general no change, with the exception of one case with positive Wassermann reaction in the CSF, in which 50% increase was observed. A few secondary syphilis cases not appearing in this table did not show a clear-cut result. In early latent syphilis there was usually a definite increase in the incubated system. Late latent syphilis showed no change as a rule; very rarely a moderate increase. A symtomatic neurosyphilis and meningovascular type of neurosyphilis exhibited either slight changes or none. Neurosyphilis, tabes, paresis, and taboparesis showed considerable increase and sometimes extremely high values were encountered.

Table I, Group E included "convulsive disorders". The genuine epilepsy cases of this group exhibited quite high autolysis. This was of special significance since the routine clinical laboratory reactions are negative in these CSF's. Specimens from patients with "symptomatic convulsions" showed either slight increase or slight decrease.

Table I, group F tabulates miscellaneous neurological conditions. In the single case of porphyriasis very high autolysis was found. Spinal fluids of brain tumor patients showed definite changes, but not very high phosphorus increase. In post-traumatic cases uniformly high phosphorus increase was found after incubation. The CSF of intracerebral haemorrhage patients showed no definite changes.

Table II gives a general picture of the distribution and degree of the phosphorus changes. As seen in Group *a*, the increase in normals is well under 10% after 24 hr. incubation. Most of the neurologically normal cases show an increase of less than 50% and generally less than 25%. Exceptionally high values are found in some upper respiratory infections ranging from 100–150% increase in a very few instances (Table II, Group *b*). The bulk of the meningitis cases is in this range, between 100–200% increase. Lower values are rare (Group *c*). The great majority of poliomyelitis cases remain under 10% (Group *d*). In the large syphilitic group (*e*) the values ranged from 10% in the early cases to 250% in neurosyphilis specimens. Genuine epilepsy cases range between 50 and 100%, symptomatic convulsions show a marked difference ranging from 0 to 25%. The miscellaneous group shows a few high values especially the post-traumatic samples in which values from 50–100% increase are seen.

The effect of the time factor of incubation was investigated. The 24 hr. interval gave the most uniform results; in the 48 hr., the type of curve may differ (Fig. 1). The temperature routinely used was 37° C., but there have

been assays which showed very high temperature optima (Fig. 2). The reversion of the hydrolytic process or synthesis, which was exceptionally observed in some 48 hr. curves at 37° C. (Fig. 1), is not very rare in many high temperature assays, two of which are shown (Fig. 2A). The effect of Mg++ activation was generally detectable; Ca++ activation sometimes gave high values.

Acid soluble total phosphorus determinations were made on a large number of specimens and definite increase of these values was found after incubation (Table IV). The significance of this finding will be further discussed. Although the pH values were found to be optimal in the acid range, we arbitrarily chose

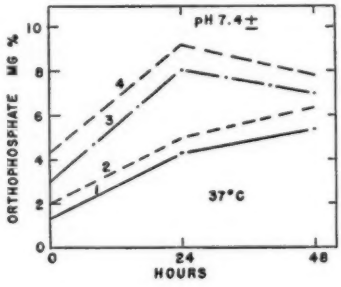

FIG. 1. EFFECT OF TIME

1 M.S. MUMPS ENCEPHALITIS
2 A.S. MGOCOCCAL MGITIS
3 M.B. NEUROSYPHILIS
4 F.D. STREPTOCOCC. MGITIS

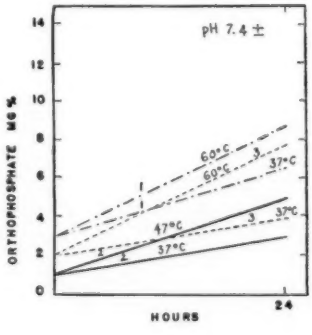

FIG. 2. EFFECT OF TEMPERATURE

1 D.L. NEUROSYPHILIS
2 L.P. INFLU. MENINGITIS
3 E.R. " "

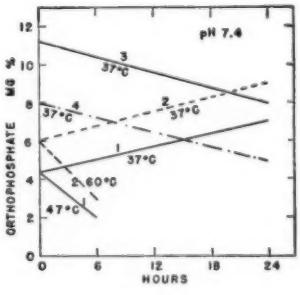

FIG. 2A. EFFECT OF TEMPERATURE

1 G.M. MGOCCAL MGITIS
2 E.H. BRAIN ABCESS
3 L.N. HYDROCEPHALUS
4 M.S. E-COLI MGITIS

TABLE III

ANALYSIS OF POLIOMYELITIS CASES

Clinical form	No. of cases	% of cases with no change	% of cases with change
Poliomyelitis	140	82	18
Nonparalytic polio	70	75	25
Paralytic polio	36	88	12
Bulbar polio	30	80	20
Polioencephalitis	4		0

TABLE IV

INORGANIC AND TOTAL ACID SOLUBLE PHOSPHORUS CHANGES IN THE SAME SPECIMENS

Group	Inorganic phosphorus, mgm. %		Total acid soluble phosphorus, mgm. %	
	0 value	End value	0 value	End value
D.E. Neurosyphilis	2.0	4.0	3.7	7.0
F.A. Meningomeningitis	2.6	4.8	4.2	8.8

as a routine ranges similar to the milieu of the body. The osmotic pressure of the buffer had no effect on the autolysis; the phosphorus change in physiological buffer was the same as that in buffer without physiological saline.

Discussion

On incubation of CSF the changes in inorganic phosphorus levels indicate hydrolysis of phosphorus-containing organic substances. This is corroborated by our finding of simultaneous increase in inorganic and in total acid soluble phosphorus in the same specimens (Table IV). The data presented by various authors describing organic phosphorus compounds in CSF will be discussed later. The individual variations in the phosphorus level of the CSF (20) and the relationship between serum and CSF inorganic phosphorus concentrations have been described by others (13). Enzymes of the CSF have been demonstrated by Kafka (10), Kaplan (11, 12), Kovacs (16), and others. The interaction of the organically bound phosphate as substrate and of the enzymes results in autolysis measured and reflected by phosphorus changes. If one or both of these factors is absent there is no autolysis, as is the case in normal or neurologically normal CSF's. The rare exceptions found in the neurologically normal group cannot be satisfactorily accounted for, except for the possibility of errors in diagnosis. In meningitides both factors are present, as evidenced by the phosphorus changes. The decrease, when it occurs, after incubation is an indication of synthesis (1, 8). The presence

of microorganisms may be the explanation of this synthesis in the *E. coli* meningitis specimens (Table I). The factor of lowered osmotic pressure as a cause of autolysis can be excluded on the basis of our assays. Physiological and nonphysiological buffers were used for incubation of the same CSF and the range of phosphorus change was the same in each case.

The absence of autolysis in the spinal fluid of poliomyelitis cases is a perplexing finding. It cannot be due to the absence of enzymes, since Lyon (19) described cytolysis and Colling and Rossiter (3) demonstrated intense phosphatase activity *in vitro*. The absence of phosphorus release in this group during the assays for autolysis suggests that cytolysis (19) is not followed by the liberation of inorganic phosphorus. Pleocytosis and protein increase on the other hand could serve as a suitable substrate for autolytical enzyme action in the noncentrifuged CSF's. The increase of proteases in the CSF of poliomyelitis was described by Kaplan *et al.* (11). Under favorable circumstances as with incubation, the proteins present, which include enzymes, are digested. The finding of Howe *et al.* (9), that cytochrome oxidases may be absent or low in monkey spinal cord at the height of chromatolysis, is somewhat analogous to our autolysis findings. That is, the absence of these or of other enzyme systems or their inhibition *in situ* or in body fluids may be a cause of the lack of autolysis. The exceptions encountered in this group of 140 persons may be explained in the following way. In nonparalytic disease the possibility of diagnostic error is great. The isolation of virus was not attempted in our series and thus essentially the diagnosis of poliomyelitis depended ultimately upon either autopsy findings or clinical paralysis; 12% of the paralytic group did not conform to the general pattern. Hence one is tempted to conclude that the exceptions found in nonparalytic and bulbar cases indicate that the majority of these were not poliomyelitis. Perhaps closer cooperation between the laboratory and clinic could throw much light on these exceptional cases.

In early syphilis it is unusual to find autolysis. Neurosyphilis cases show definite autolytic changes. Early latent specimens exhibit high phosphorus increase in contrast to late latent samples. This agrees with the clinical conception that the late latent type is a quiescent process and that early latent syphilis on the other hand is a more serious form from the point of view of prognosis. This clinical difference is seen in our results also. The presence of phosphatides and their derivatives in the CSF of syphilitic patients was claimed by Donath (4), Kafka (10), and Hauptmann (7). The enzymes in these CSF's have been described by Kafka (10), Colling and Rossiter (2), and others. The action of enzymes on the substances present can be followed by the phosphorus changes described.

Knauer and Heidrich (15) found an increase of phospholipids in the CSF of epilepsy patients. Spiegel-Adolphe (22) demonstrated nucleic acid in the CSF of electro-shocked dogs, and nucleoproteins in the CSF in convulsive disorders and post-traumatic cases (23). This author showed that these substances disappeared on standing at room temperature and on incubation

(24, 25). We were able to corroborate this finding in a few instances spectrophotometrically (17) and hence we are of the opinion that the phosphorus increase noted by us in this group resulted from the breaking down of nucleic acids by autolysis. The whole process in the post-traumatic cases may be associated with the repair of nerve cells damaged by concussion. The pathologic changes in this condition were described by Windle *et al.* (27) as chromatolysis of the neurons. The high autolysis found in our single case of porphyriasis may have resulted from extensive destruction of the nervous system.

Various findings, like different temperature optima, simultaneous increase of inorganic and total acid soluble phosphorus, etc., suggest the presence and activity of different enzyme systems during autolysis in the CSF. The same was postulated by us in neurolysis previously reported in this journal (16). We found in addition to phosphomonoesterases, nucleases and lecithinases in the CSF specimens (17) and hope to publish our results in the near future.

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